An Investigation into Bioactive Proteins and Their Changes During Imbibition, Germination and Development of Red Kidney Bean Seeds (*Phaseolus vulgaris* L.)

A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of

Philosophy in Plant Biotechnology at the University of Canterbury

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2011

Acknowledgments

I would like to extend my deepest gratitude to my supervisor Dr David Leung for all his support, precious help throughout my training and his friendship and patience during difficult times I experienced. He deserves special acknowledgement for his great supervision and the many things I learnt from his knowledge. His enthusiasm and encouragement stimulated me to develop my scientific brain and think differently. Whenever troubles struck in my professional and personal life he treated me as a friend rather than a student. I'll be always thankful for your guidance and kindness. My special thanks go to my co-supervisor, Associate Professor Tony Cole, for his bright viewpoints and useful comments about my project. His useful scientific discussion and insight on technical points are much appreciated.

I would like to express my appreciation to University of Canterbury technical staff, for their technical support, Dave Conder, Nicole Lauren-Manuera, Jan McKenzie, Craig Galilee, Jackie Healy, Manfred Ingerfeld and Matt Walters. I would also like to thank all my lab mates and friends, Tony Joseph, Ing chia Phang, Seetha, Jimmy Thomas and Josh Van Vianen for their friendship and support during my PhD.

I am also very grateful to our collaborator, Dr Fabio Apone, who kindly supplied hyacinth bean FRIL and instructions on how to pack a mannose-agarose column. My warmest thank to my lovely partner who was patient and supportive during my difficult times.

During the writing of this thesis a spate of earthquakes resulted in damages to buildings and 182 people lost their lives. It was a pretty traumatic experience in that time, living and writing under stress while the earth was shaking periodically. I would like to dedicate this work to victims of Christchurch's earthquake who lost their lives.

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List of abbreviations, symbols and nomenclature

ABA	Abscisic Acid
AFPs	Antifungal Proteins
α-AI	α-Amylase Inhibitor
AIU	α-Amylase Inhibitor Unit
ASU	α-Amylase Stimulator Unit
AU	Amylase Unit
BSA	Bovine Serum Albumin
CIE	Conidiation Inducing Effect
CM-Chitin-RBV	Methyl-Chitin-Remazol Brilliant Violet
CM-Curdlan-RBB	Carboxy Methyl linked with the dye Remazol Brilliant Blue
CM-Sepharose	Carboxymethyl-Sepharose
CU	Chitinase Unit
DHN	1,8-Dihydroxynaphthalene
DVS	Divinyl-Sulphone
GU	β-1, 3- Glucanase Unit
MALDI	Matrix Assisted Laser Desorption/Ionization
MBL	Mannose-Binding Lectin
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PEG	Polyethylene Glycol
PHA	Phytohemagglutinin
PHA-E	Erythroagglutinating Phytohemagglutinin
PHA-L	Leucoagglutinating Phytohemagglutinin
PPA	Porcine Pancreatic α-Amylase
PR Proteins	Pathogenesis-Related Proteins
PU	Protease Unit
PvFRIL	*Phaseolus vulgaris* Fetal Liver Tyrosine Kinase 3- Receptor Interacting Lectin
SDS-PAGE	Sodium Dodecyl Sulphate

	Polyacryamide Gel Electrophoresis
TIU	Trypsin Inhibitor Unit
UV	Ultra Violet
WGA	Wheat Germ Agglutinin
YPG	Yeast Extract, Peptone, Glucose Medium

Publications arising from this thesis

- Leung, D.W.M., Alizadeh, H. 2011. Extending the benefits of antifungal proteins from plants. In: Science against microbial pathogens: communicating current research and technological advances (microbiology book series). Méndez-Vilas, A. (ed.). Formatex Research Centre, Badajoz, Spain; 2: 1236-1243. (**Chapter in book**, from chapter 1 & 3 of this thesis).

- Alizadeh, H., Leung, D.W.M., Cole, A. L J. 2011. Conidiogenic effects of mannose-binding lectins isolated from cotyledons of red kidney bean (*Phaseolus vulgaris*) on *Alternaria alternata*. Phytochemistry 72: 94-99. (**Journal article**, from chapter 5 of this thesis).

- Alizadeh, H., Leung, D.W.M. 2011. Improved rapid detection of trypsin isoinhibitors using non-denaturing polyacrylamide gels with immobilized azoalbumin. Phytochemical analysis 22: 374-377. (**Journal article**, from chapter 6 of this thesis).

- Alizadeh, H., Leung, D.W.M., Cole, A. L J. 2010. Concurrent occurrence of α-amylase inhibitor and stimulator in red kidney bean seed: physiological implications. Biologia Plantarum, 54 (1): 195-197. (**Journal article**, from chapter 4 of this thesis).

- Leung, D.W.M., Alizadeh, H. 2009. Lessons from Evolution of α-Amylase Inhibitor Gene in Common Bean (*Phaseolus vulgaris*) for the

Public Concern about Genetic Engineering of Crop Plants. The International Journal of Science in Society, 1 (2): 189-194. (**Journal article**, from chapter 4 of this thesis).

Abstract

Red kidney bean seeds (*Phaseolus vulgaris*) contain a variety of bioactive proteins including lectins, enzyme inhibitors, hydrolytic enzymes and antifungal proteins. The aim of this research was to investigate activities of selected low pH and heat-stable bioactive proteins extracted from different parts of red kidney bean seed, seedling and pod as well as seed and root exudates.

Crude red kidney bean seed extracts inhibited growth of *Alternaria alternata* as well as its protease activity, but not its amylase activity. A protein with inhibitory activity against growth of *A. alternata* was purified from extracts of the red kidney bean cotyledons and embryonic axis. This purified bean protein was devoid of chitinase and β-1, 3- glucanase activities. Also, it did not inhibit porcine pancreatic α-amylase, bovine trypsin, amylase and protease of *A. alternata* suggesting that the antifungal activity of the protein is not related to these activities.

Proteinaceous extracts of red kidney bean cotyledons induced melanin and conidia formation in mycelium of *A. alternata*. A protein responsible for this conidiation inducing effect was shown for the first time to be a mannose-binding lectin which is also known as PvFRIL (*Phaseolus vulgaris* fetal liver tyrosine kinase 3-receptor interacting lectin).

An unexpected finding was that extracts of the embryonic axis stimulated rather than inhibited porcine α-amylase activity. Phytohemagglutinin (PHA-

L in particular), co-extracted with α-amylase inhibitor from red kidney bean seeds, was implicated as an α-amylase stimulator with the potential of greatly assisting digestion of starch. In cotyledonary extracts, amylase stimulatory activity was masked by amylase inhibitory activity that was inactivated when the extracts were boiled for 10 min. An in-gel non-denaturing electrophoretic method was used to show presence of porcine α-amylase isoinhibitors in extracts of the cotyledons and embryonic axis. All other seedling parts as well as seed and root exudates had amylase stimulatory activity.

Another improved non-denaturing electrophoretic method with immobilized azoalbumin was developed for in-gel detection of isoinhibitors of bovine trypsin in seed parts. It eliminates the need for both time-consuming and labourious staining, destaining or renaturation steps used in other methods.

Accumulation of most of the selected bioactive proteins during seed development in different seed parts appeared to start at 20 days after flower abscission. The activities of these proteins decreased to lower levels after 11 days of germination. Besides these observed developmental changes, under abiotic (UV-C irradiation) and biotic (seedlings co-cultured with *A. alternata*) stress, increased activity of some of the selected bioactive proteins were detected. In conclusion, this study has contributed to a better understanding of antifungal activity and the selected bioactive proteins in extracts of red kidney bean.

Chapter 1- General Introduction and Literature Review

1.1- Overview

The legumes belong to the third largest family of higher plants and they are of second in importance in agriculture after cereal crops (Gepts *et al*., 2005). Leguminous seeds are important in human diets and are fed to animals as a cheap source of protein which has high fibre as well as vitamins and minerals but low fat contents (Muzquiz *et al*., 1999; Sathe, 2002; Shimelis and Rakshit, 2005). Additionally, they are a rich source of bioactive compounds including peroxidases, ribosome inactivating proteins, antifungal proteins, saponins, phytic acid, oligosaccharides, phenolic acids, lectins as well as different inhibitors against amylase, trypsin and chymotrypsin. When consumed by animals, insects or microbes, these compounds could have impacts on their metabolism (Lam *et al*., 1998; Muzquiz *et al*., 1999; Okafor *et al*., 2002; Sawada *et al*., 2002; Ye and Ng, 2002a; Ye *et al*., 2002; Yang *et al*., 2006; Campos-Vega *et al*., 2010) which might be negative, positive or both (Champ, 2002). Some of these compounds may play a defence role in plants against pathogens and predators (Ye and Ng, 2002a; Singh *et al*., 2006).

In leguminous seeds several bioactive proteins/peptides with putative defence roles have been reported including cyclophilin-like proteins (Ye and Ng, 2000), ribosome inactivating proteins (Lam *et al*., 1998), lectins (Ye *et al*., 2001b; Singh *et al*., 2006; Shi *et al*., 2007), peroxidases (Ye and Ng, 2002a), thaumatin-like proteins (Ye *et al*., 1999), miraculin-like proteins (Ye *et al*., 2000a), defensins (Finkina *et al*., 2008), digestive enzyme inhibitors

such as amylase and protease inhibitors (Le Berre-Anton *et al*., 1997; Garcia *et al*., 2004; Macedo *et al*., 2004; Wang *et al*., 2006), chitinases (Ye and Ng, 2005), ribonucleases (Wu *et al*., 2002), β-1, 3- glucanases (Gomes *et al*., 1996; Petruzzelli *et al*., 1999; Buchner *et al*., 2002) and lipid transfer proteins (Wang *et al*., 2004a and b). Most of these proteins have antifungal activity (AFPs) and are found in extracts of dry, mature legume seeds and seed parts (Petruzzelli *et al*., 1999; Gijzen *et al*., 2001; Buchner *et al*., 2002; Wang *et al*., 2004a; Ye and Ng, 2005; Rose *et al*., 2006; Wang and Ng, 2006a and 2007a; Yang *et al*., 2006). These proteins can also be either induced or constitutive (Slitrennikoff, 2001).

Sometimes a single species may have a combination of defence proteins (Dayler *et al*., 2005). Moreover, bifunctional bioactive proteins have been found in plants (Sancho *et al*., 2003). For instance, several trypsin inhibitors can also inhibit α-amylase (Chen *et al*., 1992). Transgenic plants expressing genes of antifungal proteins have been shown to be more resistant to fungal diseases (Chandrashekar *et al*., 2000; DeGray *et al*., 2001; Epple *et al*., 1997; Gao *et al*., 2000; Montesinos, 2007; Kelemu *et al*., 2004; Ng, 2004).
In this research, a focus was placed on bioactivity of proteins from red kidney beans (*Phaseolus vulgaris* L.) seed and seedling parts. Changes in bioactivities of selected proteins in seed parts and pods during seed development, as well as in seed and seedling parts during germination with or without exposure to biotic (co-culture with *Alternaria alternata*) and abiotic (UV irradiation) stress treatments were assessed. In the experiments concerning responses to stress, the activities in the exudates of the seed and root were also tested. In particular the bioactive proteins of interest were α-

amylase and trypsin inhibitors, chitinase and β-1, 3- glucanase. For the first two activities any opposite activities (enhancers of amylase or trypsin activities) were also investigated. Following preliminary screening, the bioactivity of the proteins on *Alternaria alternata*, a fungus which was selected for further study from among different fungi supplied by the microbiology lab at the University of Canterbury, was also investigated.

1.2- Literature review

1.2.1- Taxonomy and botanical description of common bean at a glance

The worldwide common bean (*Phaseolus vulgaris* L.) is an important food crop cultivated and consumed more than other legumes and especially in developing countries it is a major source of dietary protein (Broughton *et al*., 2003; Villavicencio *et al*., 2000; Nergiz and Gokgoz, 2007). Numerous varieties of kidney bean differ in seed shape, size and colour. Variation of seed coat colour (black, red, brown and white) is determined by polyphenolic compounds (Choung *et al*., 2003; Aparicio-Fernandez *et al*., 2005). Loss of bean crops can be caused by biotic and abiotic stresses including insect pests, diseases, drought and low-soil fertility (Broughton *et al*., 2003).

The best character that can be applied to define common beans is "versatility". Common beans show a wide range of vegetative period, adaptation of plant types to diverse climatic conditions (Gepts and Debouck, 1991). Different species and cultivars of beans can grow from sea level in continental Europe and USA to above 3000 metres in Andean South America as well as from 52^0 north latitude to 32^0 south latitude (Graham and

Ranalli, 1997; Ozturk *et al*., 2009). The common bean (2n=22) is also known by several different names such as kidney, French, haricot, navy, field, pinto, marrow, China, snap, frijole, wax, string, white and black bean. All the chromosomes of the common bean have been identified and they are extremely small (Sathe, 2002; Papa and Gepts, 2003; Miklas and Singh, 2007). The common bean is a predominantly self-pollinated species which belongs to the legume family of Fabaceae (Leguminosae) comprising 643 genera and 18,000 species (Broughton *et al*., 2003; Miklas and Singh, 2007). This family is further divided into subfamilies, tribes, subtribes and genera. The genus *Phaseolus* with approximately 75 species belongs to the subfamily Papilionoideae, tribe Phaseoleae and subtribe Phaseolinae (Isely and Polhill, 1980; Broughton *et al*., 2003; Schwartz *et al*., 2005; Miklas and Singh, 2007; Mercado-Ruaro *et al*., 2009). Cultivated forms vary morphologically from bushes to climbers and are herbaceous annuals (Graham and Ranalli, 1997; Broughton *et al*., 2003). Extensive and considerable details of botanical descriptions can be found in Nasser *et al*. (2010a and b). Two types of leaves are developed in *P. vulgaris*: 1- primary leaves (two opposite prophylls) which are unifoliolate and the first two foliage leaves formed after germination, and 2- leaves formed at later developmental stages which are trifoliolate (three leaflets) compound leaves with an alternate arrangement on the stem and branches (Schwartz *et al*., 2005; Nasser *et al*., 2010b). The main stem of a seedling comprising hypocotyl and epicotyl (below and above the cotyledons) develops from the embryonic axis. Cotyledons stay on the stem for about two weeks after seed imbibition (Gepts and Debouk, 1991). The root system initially consists of a prominent tap root while adventitious and lateral roots emerge later which sometimes bear nodules containing rhizobia (Nasser *et al*., 2010a). Flowers

are perfect, zygomorphic with five petals, a single multi-ovuled ovary, a stigma, a long style and ten stamens, one of which is free and the rest of them are fused. Immature pods are green and turn yellow when mature. Pods are long, linear and slender with two valves. Seeds are large with two cotyledons, embryonic axis and seed coat (testa) account for 90, 9 and 1% of seed dry weight, respectively. Germination is epigeal as the hypocotyl elongates and pushes the cotyledons above soil surface (Schwartz *et al*., 2005; Nasser *et al*., 2010a).

Different pathogens including fungi, bacteria, nematodes, viruses and phytoplasmas can attack the common bean. Most diseases in bean are caused by fungi. Moreover, beans can be adversely affected by a number of abiotic stressors (Schwartz *et al*., 2005).

Red kidney bean seeds contain a number of bioactive proteins including chitinase, β-1,3- glucanase, amylase and trypsin inhibitors. The last two aforementioned bioactive proteins can diminish starch and protein digestibility, respectively and chitinase and β-1, 3- glucanase are able to degrade major structural polysaccharides of fungal cell walls. All these proteins could have antifungal activity and some have been shown to be involved in plant defence.

1.2.2- Fungi and plant diseases

Fungi are a group of heterotrophic eukaryotes which are devoid of chlorophyll and have an amazing ability to grow on almost any surface including skin, leaves and even bathroom tiles (Selitrennikoff, 2001;

Borkovich *et al*., 2004). They are important in degrading biological matter, some of which are consumed in the form of edible mushrooms or blue cheese and some are associated with plant roots (over 90% of plants have mycorrhiza) as mutualists to help plants to scavenge minerals from nutrient-poor soils. They can also be regarded as the most biotechnologically useful organisms which are used in the production of economically important compounds such as antibiotics or other pharmaceuticals as well as different enzymes. On the other hand, they are also able to colonize the human body, plants and animals and use them as a substrate for growth (Alexopoulos *et al*., 1996; Carlile *et al*., 2001; Selitrennikoff, 2001; Borkovich *et al*., 2004). Even though no more than 100,000 species have been described, the number of fungi is estimated to be about 1.5 million (Hawksworth, 2001; Dichinson, 2003; Stephenson, 2010). The majority of the described fungi are saprophytes. More than 10,000 cause diseases on plants and a few are known to be pathogens in humans, animals, fish and insects (Dichinson, 2003). Fungal infection can reduce crop yield and is one of the main challenges in productive agriculture (Agrios, 1988).

Morphologically most fungi are filamentous while some are observed as yeasts and primitive ones (such as the chytridomycetes) which attach to substrate as individual cells or dichotomous branched chains of cells with their rhizoids (Walker and White, 2005). They may release digestive enzymes (literally called ‘exoenzymes’) into their environment to degrade large and complex molecules in food substrates to simpler molecules which can then be taken up through the hyphal wall into the cytoplasm (Stephenson, 2010).

1.2.2.1- Conidiation and melanization in fungi

Reproduction in fungi can occur either sexually or asexually but it seems that some species (imperfect fungi) have lost their sexual reproduction capability. Sexual reproduction can be characterized by union of two nuclei, while in asexual reproduction reproductive cells (such as spores) are produced without fusion of nuclei and obtained from mitosis (mitospores) (Alexopoulos *et al*., 1996; Fischer and Kues, 2006; Stephenson, 2010). Asexual reproduction of many fungi is considered more important because it can occur several times during a season producing more propagules or spores in comparison with sexual reproduction of those that occur only once per year. Moreover, asexual spores can serve as resting bodies when the conditions are unfavourable for fungi or in mating with other individuals. Various types of asexual spores as well as different modes of spore production have evolved in fungi and they are used as criteria in fungal taxonomy (Class Deuteromycota has been defined based on fungal asexuality) (Fischer and Kues, 2006). If asexual spores appear at the tips or side of hyphae they are called conidia (Alexopoulos *et al*., 1996). Study on fungal conidiation is important as conidia can be used in agriculture, food or pharmaceutical industries (Alexopoulos *et al*., 1996). Some fungi form spores only when they grow on a substrate surface and not in a submerged liquid culture (Fischer and Kues, 2006).

Conidiation in fungi can be induced by a variety of environmental biotic and abiotic factors and stresses (Pascual *et al*., 1997; Masangkay *et al*., 2000; Yoshida and Shirata, 2000; Chovanec *et al*., 2001; Mills *et al*., 2004; Fischer and Kues, 2006; Carvalho *et al*., 2008; Nemcovic *et al*., 2008; Antony-Babu and Singleton, 2009; Zhang *et al*., 2009b. In some fungi, during spore

formation fungal melanins (dark brown pigments) are synthesized and deposited in cell walls. Involvement of melanin in sexual and asexual spore formation of fungi has been described in previous studies (Calvo *et al*., 2002). Pyroquilon, an inhibitor of melanin synthesis, inhibited spore formation in *Magnaporthe grisea* significantly (Uehara *et al*., 1995). Fungal growth, conidiation and melanin production were remarkably reduced in *Cryphonectria parasitica*, a fungus known as chestnut blight agent, when *cpg1* and *cpg2*, which encode α- and β-subunits of a G protein, respectively, were deleted. In *Alternaria alternata* melanin is important in spore development and disruption of *brm2*, a melanin biosynthethesis gene, caused reduced melanin production in the fungus. At the same time the conidia were smaller and sensitive to UV light in comparison with those of the wild type (Calvo *et al*., 2002).

Melanins are difficult to study as they defy structural and biochemical analysis. They are an important virulence factor in some fungi and they contribute to fungal protection against UV light as well as biotic and abiotic environmental factors and stresses (Calvo *et al*., 2002; Nosanchuk and Casadevall, 2003; Plonka and Grabacka, 2006; Schiave *et al*., 2009). Melanization has also reduced fungal susceptibility to enzymatic degradation, antifungal drugs and toxic metals (Gomez and Nosanchuk, 2003; Butler *et al*., 2009). Melanin production is necessary for the survival of *Cochliobolus heterotrophus*, a maize pathogen based on fitness studies of albino spore mutants. Melanized fungi have been reported to be more resistant than those of their non-melanized mutants and they have also less porous in their cell walls (Butler *et al*., 2005; Jacobson and Ikeda, 2005). Even though a critical role for melanization of appressoria in some fungi has

been indicated (Gachomo *et al*., 2010) *Alternaria* species produce appressoria without melanin and still retain their pathogenicity (Thomma, 2003). To date, no investigation has been carried out on inducing activity of plant proteins on either fungal melanization or conidiation. It could be a new area of research to assess the effect of bioactive proteins on conidiation.

1.2.2.2- *Alternaria alternata* (Fries) Keissler, a cosmopolitan fungus

Alternaria diseases are considered the most common diseases of many different kinds of plants. They affect flowers, fruits, stems and leaves of annual plants as well as some parts of some trees including apple and citrus. Visible symptoms usually include blights and leaf spots but collar, tuber and fruit rots as well as seedlings damping off have also been reported (Agrios, 1988).

The conidia and conidiophores of the genus *Alternaria* are dark. The conidia have septa (both cross and longitudinal) and are variously shaped, inversely clavate to ovoid or elliptical and can be easily detached. Conidiophores are mostly simple, sympodial or determinate, short and erect that bear single or branched chains of conidia (Agrios, 1988; Barnett and Hunter, 1999). Identification of the *Alternaria* species is based on morphological characteristics such as size, shape and septation of conidia (Muller, 1991; Bajwa *et al*., 2010). *Alternaria* species have two major features: melanin production which can be especially observed in spores and host-specific toxin production in pathogenic species (Thomma, 2003).

There are seven pathotypes of *Alternaria alternata* that cause disease on different plants by different host-specific toxin production (Furukawa and

Kishi, 2001; Ito *et al*., 2004). It has been reported as a pathogen of *Phaseolus vulgaris* causing seed pod blemishes and severe leaf spots (Russell and Brown, 1977). The fungus has also been investigated and used as a biocontrol agent against weeds and insects (Masangkay *et al*., 2000; Christias *et al*., 2001; Babu *et al*., 2002; Hatzipapas *et al*., 2002; El-Morsy *et al*., 2006). *A. alternata* accumulates 1,8-dihydroxynaphthalene (DHN) melanin in the cell walls of hyphae and conidia (Kimura and Tsuge, 1993) and is able to secrete proteases and amylases into the culture medium (Patil and Shastri, 1985; Abdel-Sater and Ismail, 1993; Chung and Hwang, 1996; Dunaevskii *et al*., 1996; Shafique *et al*., 2010). To date, no research has been conducted to assess any plant-derived proteinaceous inhibitors against these hydrolytic enzymes. *A. alternata* is also known to produce abscisic acid (ABA) (Crocoll *et al*., 1991), which is a plant growth regulator. ABA has been implicated in susceptibility of plants to pathogens but recently it has been suggested that it can render plants more resistant (Flors, *et al*., 2005). Therefore, ABA's role in plant-pathogen interactions is controversial.

1.2.3- Defence and antifungal proteins in plants and their distribution

Plants, unlike animals, are unable to escape dangers and have developed an arsenal of defence mechanisms to combat pests and pathogens or other biotic and abiotic stressors. The first line of defence is structural characteristics or physical barriers that prevent pathogens entering plants and the second is production of biochemical compounds before or after infection which can be either low-molecular weight natural products or proteins. These substances including enzyme inhibitors or pathogen-targeted hydrolytic enzymes can prevent pathogen attack or inhibit growth of pathogens. Cereal seeds, for example, comprise a testa-pericarp layer with phenolic compounds.

Additionally, the storage tissue of these seeds contains inhibitors of microbial and mammalian enzymes (Leah *et al*., 1991). Different defence-related proteins have been reported which combat foreign invaders. Some of them (pathogenesis-related, or PR proteins) may appear in plants as they respond to pathogen attack, mechanical and chemical agents or different environmental stressors. Induced disease resistance mediated by PR-proteins in plants has been reported as non-specific. It means that regardless of the type of inducer, once induced the plant displays resistance to a broad spectrum of pathogens, not just the one that initially caused induction. Some natural components, for example, fungal and bacterial proteins, polysaccharides, lipoproteins, viral coat proteins and yeast RNA as well as synthetic molecules including salicylic acid, polyacrylic acid and 2-chloroethylphosphonic acid are able to induce disease resistance in plants. Some proteins are present in plants before infection and expressed constitutively. Examples of these include hydrolytic enzymes of plants (such as glucanases and chitinases) that can damage the pathogen cell wall and inhibitors of hydrolytic enzymes of pathogens (Agrios, 1988). Defence-related proteins may act indirectly against plant pathogens. Glycine-rich proteins or hydroxyproline-rich glycoproteins, for example, alter or strengthen plant cell walls and consequently create a physical barrier against pathogens (Vance *et al*., 1980; Hammerschmidt *et al*., 1984). However, some proteins destroy the invading pathogens directly. Proteinase and amylase inhibitors, chitinases, β-1, 3- glucanases, toxic proteins like lectins and other different groups of proteins like defensins can be placed in this group of defence-related protein (Gomes *et al*., 1996; Petruzzelli *et al*.,

1999; Ye *et al*., 2001b; Buchner *et al*., 2002; Figueira *et al*., 2003b; Svensson *et al*., 2004; Wang *et al*., 2006; Finkina *et al*., 2008).

Antifungal proteins (AFPs), ubiquitous components in different plant parts, belong to a group of plant defence proteins that have a broad spectrum of biological activity and play a key role in plant defence against pathogenic organisms by preventing or limiting spread of fungi (De Wit, 1992). They can serve to protect plants from devastating damage caused by fungal pathogens and consequently prevent economic losses (Wang and Ng, 2006a).

Occurrence of AFPs in seeds might be involved in protecting seeds during resting or storage and germination (Chilosi *et al*., 2000; Nobrega *et al*., 2005). These natural defence mechanisms are necessary as seeds are likely to encounter plenty of soil-borne pathogenic organisms. At the imbibitional stage, seed coat can represent a physical and chemical barrier against aggressors until there are favourable conditions for seed germination (Ramos *et al*., 1998). In germinating seeds, the growing radicle ruptures the tissue of endosperm and makes the embryo accessible to pathogens (Witmer *et al*., 2003). Therefore, a post-germination defence strategy can protect germinating seeds when physical barriers are unable to protect the embryo from pathogenic organisms (Flach *et al*., 1992; Rose *et al*., 2006; Yang *et al*., 2006).

AFPs have been isolated from different tissues and parts of plants, suggesting their involvement in plant defence. Some of them have been reported from bulbs (Wang and Ng, 2001a; Deepak *et al*., 2003), whole

seeds, seed parts (Terras *et al*., 1995; Guo *et al*., 1999; Petruzzelli *et al*., 1999; Agizzio *et al*., 2003; Santos *et al*., 2008), leaves (Khai Huynh *et al*., 1996; Yang and Gong, 2002; Ghosh *et al*., 2004), roots (Wang and Ng 2000b; Lam and Ng, 2002), shoots (Wang and Ng, 2003) and bark (Huang *et al*., 2002). AFPs can be grouped into different classes according to their activities and structures (Ng, 2004). These groups include chitinases (Benhamou *et al*., 1993; Gijzen *et al*., 2001; Ye and Ng, 2005), glucanases (Leah *et al*., 1991; Buchner *et al*., 2002), protease inhibitors (Wang *et al*., 2006), thaumatin-like proteins (Ye *et al*., 1999), ribosome inactivating proteins (Lam *et al*., 1998), embryo-abundant proteins (Wang and Ng, 2000a), cyclophilin-like proteins (Ye and Ng, 2002b), miraculin-like proteins (Ye *et al*., 2000a), novel proteins (Wang and Ng, 2001b; Ye *et al*., 2002), allergen-like proteins (Ye and Ng, 2001a), chitin-binding proteins (Huang *et al*., 2000), ribonucleases (Ng and Wang, 2000; Wu *et al*., 2002), deoxyribonucleases (Wang and Ng, 2001c), lectins (Sitohy *et al*., 2007), defensin-like peptides (Wong and Ng, 2005b), defensins (Thomma *et al*., 2002), thionins (Terras *et al*., 1993), peroxidases (Estela da Silva and Franco, 2000; Ye and Ng, 2002a), lysozymes (Wang *et al*., 2005), non-specific lipid transfer proteins (Wang *et al*., 2004a and b), chitinase-like proteins (Lam *et al*., 2000; Lam and Ng, 2001; Ye *et al*., 2000b; Ye and Ng, 2002c), cysteine-rich proteins (Terras *et al*., 1995) and peptides (Almeida *et al*., 2000; Ye and Ng, 2001b). These proteins have been named based on their structures (e.g., cysteine-rich), mode of action (e.g., chitinases) or their similarities to other known proteins (e.g., miraculin-like proteins). Unfortunately a standardised nomenclature based on structure or other unifying properties of these proteins has not been proposed (Slitrennikoff, 2001).

1.2.4- Mode of actions of AFPs

Different mechanisms of actions of AFPs have been reported including formation of membrane channels, degradation of polymers in the fungal cell wall or disruption of cellular ribosomes but the precise mechanisms by which many of these proteins to inhibit fungal growth remains unknown (Hermanova *et al*., 2006). It has been demonstrated that growth inhibition of *Aspergillus flavus* by a plant-derived trypsin inhibitor may be linked to fungal amylase inhibition by the same protein and consequently limiting the availability of simple sugars for fungal growth (Chen *et al*., 1999). One possible mechanism of PR-5 in inhibition of fungal growth was reported to be associated with formation of pores in fungal plasma membrane and consequently leading to its rupture and permeabilization (Batalia *et al*., 1996). Lectins can inhibit protein synthesis by binding to ribosomes (Gonzalez de Mejia and Prisecaru, 2005). Chitinases and β-1, 3- glucanases degrade the fungal cell wall and therefore inhibit fungal growth (Mauch *et al*., 1988; Joosten and De Wit, 1989; Kuzniak and Urbanek, 1993). More recently, research on the mode of action of chitinase on four different fungi demonstrated that chitinase has different antifungal activities against each fungal species. This was correlated with the proportion of chitin in the cell wall of the different fungi and their surface microstructure (Yan *et al*., 2008). Fungicidal activity of thionins, plant defensins and osmotin has also been attributed to their effect on fungal membranes (Abad *et al*., 1996; Pshenichnov *et al*., 2005).

1.2.5- Changes in AFPs during seed germination and development

Seeds are rich in food reserves which are enzymatically degraded during germination to support seedling growth. Some proteins such as inhibitors of hydrolytic enzymes may protect germinating seeds against fungal and insect pest attack (Muzquiz *et al*., 2004). There are reports about changes in trypsin inhibitor activity during germination in different seeds. Trypsin inhibitor activity in pinto beans increased by more than 39% after 6 days of germination while it did not decrease significantly during germination in Navy beans (Chang and Harrold, 1988). During 10 days of germination, trypsin inhibitor activity decreased by 30% in French beans (Nielsen and Liener, 1988). A significant decrease (52.5%) was observed in the content of trypsin inhibitor during germination of white bean seeds (Sangronis and Machado, 2007). In cotyledons of two varieties of *Vicia faba* trypsin inhibitor content increased relative to total proteins suggesting that trypsin inhibitor proteins were not hydrolysed while other proteins were (Muzquiz *et al*., 2004). Amylase inhibitory activity in great Northern beans was decreased by 61% after 5 days of germination (Savelkoul *et al*., 1992).

At 20 days after flowering no trypsin inhibitory activity was detectable in two varieties of chickpeas while one of the varieties (Annigeri) showed the highest activity at 40 days after flowering (Harsulkar *et al*., 1997). In white bean seeds, papain inhibitory activity was detected at early maturation stages during seed development (Santino *et al*., 1998). Different levels of trypsin inhibitor content in four different genotypes of Indian soybean at very early stage of seed development were also observed (Kumar *et al*., 2005).

Chitinases accumulate developmentally in several seed species and may protect them against chitin-containing pathogenic fungi (Gomez *et al*., 2002).

1.2.6- Changes in AFPs in response to exposure to biotic and abiotic stressors during seed germination

Plants are frequently exposed to environmental conditions that affect their growth, development and productivity (Bray *et al*., 2000). The early seedling stage and seed germination are considered as the most sensitive stages to adverse environmental conditions with the highest mortality in many crops (Abdallah *et al*., 1989). Different classes of defence proteins are produced in response to stress. Pathogenesis-related proteins (PR) which are coded by host plants are induced by abiotic or biotic agents in many plant species of various families (Van Loon and Van Strien, 1999). When receptor components in inducible systems recognize the challenge, signal transduction pathways are activated that consequently cause effects on response genes and finally resistance (Katile *et al*., 2010). The protein osmotin, for example, is expressed and produced in response to either osmotic stress or pathogen attack (Singh *et al*., 1989; Abad *et al*., 1996). PR proteins have been classified into 17 families based upon enzymatic or biological activity, sharing amino acid sequences, the serological relationships and the following two criteria. Firstly, a protein must be expressed in response to pathogen exposure. Secondly, protein induction must have been shown to occur in response to a minimum of two different plant-pathogen combinations or protein expression in a single plant-pathogen combination must be confirmed by two independent laboratories. Therefore, based on these criteria proteins that are found in healthy tissues

and are homologous to PR proteins cannot be categorized as PR proteins unless they are induced by a biotic or abiotic agent. Among the 17 PR families, four families belong to chitinases including PR-3, -4, -8 and -11, one β-1, 3- glucanases (PR-2) and one protease inhibitor (PR-6). An extensive explanation about the criteria and categorization with some examples of each family can be found in Van Loon and Van Strien (1999) and Van Loon *et al*., (2006). Three classes have been considered for PR-2 proteins based on amino acid sequence analysis and all have been shown to have β-1, 3- endoglucanase activity *in vitro* (Cote *et al*., 1991; Leah *et al*., 1991; Selitrennikoff, 2001). Chitinases and β-1, 3- glucanases have been induced in plant leaves in response to fungal inoculation (Muthukrishnan *et al*., 2001) and PR-10 has also been expressed in sorghum as a response to fungal infection (Lo *et al*., 1999). When bean seedlings were inoculated with a non-pathogenic binucleate *Rhizoctonia* species 48 h prior to contamination by either *Rhizoctonia solani* or *Colletotrichum lindemuthianum*, the systemic resistance was induced and the seedlings showed less disease severity and fewer necrotic lesions in comparison with seedlings not inoculated with the non-pathogenic species (Xue *et al*., 1998). Expression of a cDNA encoding a cysteine protease inhibitor gradually decreased during germination of amaranth seeds and its expression increased in stems and roots in response to abiotic stressors (Valdes-Rodriguez *et al*., 2007).

1.2.7- Application of AFPs

The economic costs of fungal infections are large and ongoing, creating a need for novel antifungal agents that are safer and more environmentally friendly as desirable control measures (Montesinos, 2007; Munoz *et al*., 2007; Meyer, 2008). Application of an antifungal protein from *Aspergillus giganteus* on rice leaves has been successful to control *Magnaporthe grisea* infection (Vila *et al*., 2001). A protease inhibitor isolated from potato sprouts reduced fungal lesions caused by *Botrytis cinerea* on tobacco leaves (Hermosa *et al*., 2006). Tomato plants were protected against *Fusarium oxysporum* when the roots were pre-incubated with an antifungal protein from *Aspergillus giganteus* (Theis *et al*., 2005). Another approach involves generation of transgenic plants expressing antifungal proteins which have resulted in significant reduction of disease symptoms (Epple *et al*., 1997; Chandrashekar *et al*., 2000; Gao *et al*., 2000; DeGray *et al*., 2001; Bieri *et al*., 2003; Kelemu *et al*., 2004; Ng, 2004; Vasconcelos and Oliveira, 2004; Dunaevskii *et al*., 2005a; Pshenichnov *et al*., 2005; Montesinos, 2007; Fiocchetti *et al*., 2008). This has been successful in carrots, potatoes and rice (Gao *et al*., 2000; Nishizawa *et al*., 1999; Punja and Raharjo, 1996). Expression of osmotin genes in transgenic potatoes has resulted in increased resistance to *Phytophthora infestanse*, a fungus that is known as the late-blight pathogen on potatoes (Zhu *et al*., 1996). Almost all PR families have been expressed in transgenic plants and these plants have become more resistant to diseases (Brogue *et al*., 1991; Alexander *et al*., 1993; Howie *et al*., 1994; Liu *et al*., 1994). Chitinases and β-1, 3- glucanases are the most attractive antifungal proteins due to their strong *in vitro* activity. Transgenic plants over-expressing chitinases or β-1, 3- glucanases demonstrated

enhanced resistance to fungal pathogens and a synergistic benefit was observed when both transgenes were present (Khan, 2002). Due to the potential of broad-spectrum resistance from use of barley chitinase with antifungal activity, the chitinase gene could be used to enhance fungal-resistance in crop plants such as tobacco, rice, clover and tea (Kirubakaran and Sakthivel, 2007).

1.2.8- Bioactive proteins studied in this research

1.2.8.1- Lectins

Lectins (from the Latin word "legere" meaning "to select"), also called agglutinins or hemagglutinins, occur widely in fungi, bacteria, plants, animals and viruses and have been studied over a century (Gonzalez de Mejia and Prisecaru, 2005; Van Damme *et al*., 2008). Plant lectins are proteins with at least one non-catalytic domain which binds reversibly to a specific mono- or oligosaccharide. The agglutination properties of lectins are attributed to their binding specificity towards sugars. A small change in the structure of binding site can change lectin specificity and consequently biological activity (Barre *et al*. 2001). These sugar binding proteins have been isolated from different parts of plants including seeds, stems, bulbs, bark, corms, tubers, flower tissues, rhizomes, phloem, roots, leaves and fruits (Sales *et al*., 2000; Ye *et al*., 2001b; Vasconcelos and Oliveira, 2004; Gonzalez de Mejia and Prisecaru, 2005). Legumes are considered to have a high content of lectins which varies from 5% dry weight in beans to 6.5% in soybean (Gonzalez de Mejia and Prisecaru, 2005; Zhang *et al*., 2009a).

The physiological roles of plant lectins are still not clear. However, their effects on other organisms have been widely investigated and based on these they should be considered as defence-related proteins (Vasconcelos and Oliveira, 2004; Van Damme *et al*., 2008). Several lectins have been discovered that are not constitutively detectable in plants as they are induced in plants by biotic or abiotic factors. For example, orysata, a mannose-specific jacalin-related lectin, was expressed in excised rice leaves after contamination with *Magnaporthe grisea* (Van Damme *et al*., 2008). Plant lectins have potent biological activities. Even though not all plant lectins are toxic, a group of them are extremely cytotoxic and play a main role in plant defence systems (Chrispeels and Raikhel, 1991; Gonzalez de Mejia and Prisecaru, 2005; Van Damme *et al*., 2008).

Legume lectins, for example, have been reported to exhibit numerous biological activities such as anti-fungal (Ye *et al*., 2001b), anti-insect (Singh *et al*., 2006), anti-tumor (Chun-Yang *et al*., 2010), anti-cancer (Gonzalez de Mejia and Prisecaru, 2005) and anti-human immunodeficiency (Ye *et al*., 2001b; Fang *et al*., 2010). Some symptoms of lectin toxicity include nausea, diarrhoea, vomiting and bloating in humans and growth inhibition in animals (Liener, 1982). Wheat germ agglutinin (WGA) was assessed for antifungal activity against *Trichoderma viride*. It was able to inhibit fungal growth and spore germination. As WGA interacts with chitin oligomers, it has been assumed that the lectin protects the seed during imbibition, germination and even early seedling growth against chitin-containing pathogens (Mirelman *et al*., 1975).

1.2.8.2- Chitinases and β-1, 3- glucanases

Plants do not have chitin, the natural substrate for chitinases, but chitinase activity is present in leaves, seeds or root extracts (Leung, 1992; Gomez *et al*., 2002). Chitin, together with β-1, 3-glucan, is the main structural component of the cell wall of some fungi (Mauch *et al*., 1988; Joosten and De Wit, 1989; Arlorio *et al*., 1992). Degradation of these structural polysaccharides of the fungal cell wall by hydrolytic enzymes such as chitinase and β-1, 3- glucanase could limit fungal growth (Mauch *et al*., 1988; Joosten and De Wit, 1989). Moreover, a degraded glycosidic fragment of the fungus may play a role as an elicitor for stress metabolite biosynthesis in the host (Leah *et al*., 1991; Lima *et al*., 2002). Antifungal activity of chitinases, particularly in combination with β-1, 3- glucanase, has been demonstrated *in vitro* (Mauch *et al*., 1988; Ignatius *et al*., 1994). Among 18 fungi tested in one experiment, either a purified chitinase or β-1, 3-glucanase from pea pods when applied separately was able to inhibit one fungus but when they were used together most fungi were inhibited (Mauch *et al*., 1988). Similar synergistic activity of chitinase and β-1, 3- glucanase from tobacco (Sela-Buurlage *et al*., 1993), cucumber (Ji and Kuc, 1996) and chitinase with thaumatin-like protein from barley (Hejgaard *et al*., 1991) have been reported.

Based on structural properties, chitinases have been divided into seven classes (Singh *et al*., 2007). They vary in size ranging from 20 to 90-KDa (Ignatius *et al*., 1994). Multiple isoforms of chitinases may be present in seeds (Collada *et al*., 1993). For instance, at least five chitinase isozymes in soybean seeds have been described (Yeboah *et al*., 1998).

Light microscopic examination disclosed hyphal tip swelling, hyphal distortions and cytoplasm leakage when *Rhizoctonia solani* was in contact with chitinase of ethylene-treated bean leaves (Benhamou *et al*., 1993). A class I chitinase was expressed in the pod, root, leaf and seed coat of soybean (Gijzen *et al*., 2001). Chitinase activity in seeds and the different seed parts (coat, cotyledons and axis) of two different bean cultivars, hard brown (*Phaseolus vulgaris* cv. Maisugata) and soft white *P. vulgaris* cv. Surattowonder), was compared. The enzyme activity was found in all the seed parts in both cultivars but at different levels. In this research, the ability of the enzyme for fungal inhibition was not investigated (Ramos *et al*., 1998). A chitinase with antifungal activity against *Rhizoctonia solani* which caused fungal cell wall disruption and cytoplasm leakage was purified from ethylene-treated bean leaves (Benhamou *et al*., 1993). Other chitinase isolated from mung bean seeds exerted antifungal activity toward *Pythium aphanidermatum*, *Sclerotium rolfsii*, *Mycosphaerella arachidicola*, *Fusarium solani* and *F. oxysporum* (Ye and Ng, 2005). A 30-KDa thermostable chitinase from *Adenanthera pavonine* seeds was isolated. This protein was also detected in roots, exudates of seeds and roots and cotyledons (Santos *et al*., 2007).

It has been reported that not all chitinases possess antifungal activity as some may have either developmental or signal transduction roles (Baek *et al*., 2001; Lima *et al*., 2002; Ye and Ng, 2005). In sorghum cultivars, for example, no association was found between resistance to grain mould and chitinase accumulation (Prom *et al*., 2005). A class I chitinase from tobacco exhibited antifungal activity against *Fusarium solani* while class II did not

(Sela-Buurlage *et al*., 1993). Other functions of chitinases are: involvement in nodule formation (Goormachtig *et al*., 1998), embryogenesis (Kragh *et al*., 1996), antifreeze activity (Hon *et al*., 1995) and involvement in some developmental processes (Lotan *et al*., 1989; Neale *et al*., 1989; Neale *et al*., 1990; Leung, 1992; Hanfrey *et al*., 1996).

Several functions have been reported for β-1, 3- glucanase in plant tissue including microsporogenesis, regulation of pollen tube growth, fertilization, callose removal, cell elongation and seed glucan degradation (Abeles and Forrence, 1970; Beffa and Meins, 1996). β-1, 3- Glucanase activity was detected in tomato endosperm but not in the embryo. The activity developed in the seeds following germination (Morohashi and Matsushima, 2000). In a study on six thaumatin-like proteins purified from different parts of tomato, cherry, barley and tobacco plants, it was shown that all proteins have glucanase activity on polymeric glucan (Grenier *et al*., 1999).

1.2.8.3- Protease inhibitors

Seeds of legumes contain proteinaceous inhibitors that may inhibit activity of microbial proteinases (Chen *et al*., 1999). Protease inhibitors have been classified into 10 families on the basis of their molecular weight, amino acid sequence, homology and cysteine contents (Campos *et al*., 2004). Of all the protease inhibitors, trypsin inhibitors are the most extensively studied (Chen *et al*., 1999) and found to be more heat-resistant than chymotrypsin inhibitors (Ee *et al*., 2008). Most of Bowman-Birk family members have been isolated from legumes and they have two reactive sites that enable

them to inhibit two proteases independently at the same time or even two molecules of a protease (Campos *et al*., 2004).

The protective role of protease inhibitors in seeds against microbial pathogens was shown in transgenic plants by expressing cystatin genes (Santino *et al*., 1998). A protease inhibitor isolated from potato inhibited phytopathogenic fungi *in vitro* (Hermosa *et al*., 2006). A Kunitz-type trypsin inhibitor with antifungal activity toward *Fusarium oxysporum* was also isolated from *Pseudostellaria heterophylla* roots (Wang and Ng, 2006b). At least five different trypsin inhibitors were isolated from *Phaseolus vulgaris* var. Kintoki bean (Tsukamoto *et al*., 1983). A trypsin-chymotrypsin inhibitor was also isolated from *P. vulgaris* (var. Kidney bean) with a molecular weight of 13000 Da (Jacob and Pattabiraman, 1986) but their activity against fungi or in response to stress was not assessed. A novel protease inhibitor with antifungal activity was isolated from mung bean seeds (Wang *et al*., 2006). A Bowman-birk-type trypsin-chymotrypsin inhibitor has also purified from broad beans (Ye *et al*., 2001a).

Proteinaceous inhibitors with additional favourable properties or with chitinase activity are potentially very useful in biotechnology and the genes coding for the proteins with a combination of these biological activities can be used to create transgenic plants with multiple activities toward fungi (Franco *et al*., 2002).

1.2.8.4- α-Amylase inhibitors and stimulators

Amylases are required by some pathogens and play an important role in plant-microbe interactions (Fakhoury and Woloshuk, 1999). Proteinaceous α-amylase inhibitors have been involved in plant defence against pathogens (Svensson *et al*., 2004) and can be grouped together based on their tertiary structures into six classes (Payan, 2004).

Plant seeds are a rich source of proteinaceous feeding deterrents including α-amylase inhibitors that inhibit α-amylases from different sources. α-Amylase inhibitors of the common bean were first described as lectin-like proteins and association with plant defence activity (Moreno and Chrispeels, 1989).
It has been suggested that the trypsin-amylase inhibitor protein could contribute to the resistance of maize to *Aspergillus flavus,* a fungal pathogen that causes ear and kernel rot disease (Chen *et al*., 1999; Fakhoury and Woloshuk, 2001; Kim *et al*., 2007). An α-amylase inhibitor (19.7 KDa) isolated from corn kernels was used to inhibit the amylase activity of *Fusarium verticillioides* and reduce conidia germination (Figueira *et al*., 2003b). A 36-KDa protein from *Lablab purpureus* inhibited growth of *Aspergillus flavus* (maize kernel pathogen) and inhibited α-amylase activity of the aforementioned fungus as well as *Helminthosporium victoriae* and *Magnaporthe grisea* (Fakhoury and Woloshuk, 2001; Kim *et al*., 2007).
Presence of a stimulator of porcine α-amylase in red kidney beans has been reported before. It was found that a tetrameric isoform of phytohemagglutinin (PHA) is able to enhance the amylase activity (You and Chang, 1992). Molecular biological studies have uncovered a fascinating relationship between the α-amylase inhibitor and phytohemagglutinins

(PHA) found in the bean seed. They are very similar at the gene sequence level differing in only a few amino acids (Young *et al*., 1999; Wato *et al*., 2000). Amylase inhibitors have been isolated, purified and characterized from different varieties and accessions of *Phaseolus vulgaris* including red, white and black kidney beans (Le Berre-Anton *et al*., 1997). Seeds of the common bean are a rich source of proteinaceous inhibitors of digestive α-amylase (Yamada *et al*., 2001). Eight variants were identified in the common bean on the basis of electrophoretic mobility and inhibitory activity against α-amylases from Mexican bean weevil (*Zabrotes subfasciatus*) and porcine pancreas (Ishimoto *et al*., 1995). Two related α-amylase inhibitors designated α-AI1 and α-AI2, with 78% sequence identity at the amino acid level, were isolated from cultivated and the wild common bean respectively (Finardi-Filho *et al*., 1996; Ishimoto and Chrispeels, 1996; Grossi de Sa *et al*., 1997; Le Berre-Anton *et al*., 1997). α-AI1 inhibited mammalian and the larval midgut α-amylases of Azuki bean and cowpea weevil but not of Mexican weevil. α-AI2 only inhibited Mexican weevil α-amylase (Ishimoto and Chrispeels, 1996). α-AI1 is closely linked with PHA while α-AI2 is linked to specific arcelin variants (Suzuki *et al*., 1995). Another isoform of α-AI is α-AI3 or α-AIL which has no amylase inhibitory activity but resembles α-AI in its amino acid sequence (Finardi-Filho *et al*., 1996). α-Amylase1 isolated from *Phaseolus vulgaris* cv. Magna inhibited neither *Fusarium* spp. nor *Sclerotinia* spp. (Kluh *et al*., 2005). In this research, the proteins of the red kidney bean with α-amylase inhibitory activity against fungal and porcine pancreatic α-amylase were studied.

1.2.9- Bifunctional proteins

Bifunctional proteins, for example, α-amylase/proteinase inhibitors are relatively common. A bifunctional α-amylase/subtilisin inhibitor was purified from rice bran. Its activity remained constant during germination and it was synthesized in the milky stage in developing seeds (Yamagata *et al*., 1998). The bifunctional trypsin/α-amylase inhibitor genes from corn were transferred into tobacco plants. Protein extracts from the transgenic tobacco plants were more inhibitory against trypsin and amylase than control plants (Masoud *et al*., 1996).

A protein with trypsin and α-amylase inhibition activities isolated from maize seeds has strong sequence similarities with thaumatin, a virus-induced pathogenesis-related (PR) protein (Bloch and Richardson, 1991). A 33330 Da bifunctional protein named "*Phaseolus vulgaris* chitinolytic α-amylase inhibitor" (PvCAI) was isolated from wild common bean seeds. This protein possesses both α-amylase inhibitory and chitinase activities and has in the range of 15-30% identity with common bean chitinases. A comparison between molecular masses of six α-amylase inhibitor classes showed that the molecular mass of this protein differs from them (Dayler *et al*., 2005). An α-amylase inhibitor isolated from *Coix lachrymal-jobi* was found to possess chitinase activity and exhibited sequence similarity with various endochitinases (Bloch and Richardson, 1991). According to our knowledge, no bifunctional protein has been reported from the cultivated common bean. Bifunctional proteins with a great potential against aggressors may be used for the production of transgenic plants.

1.3- Objectives

The study of bioactive proteins gives information on their potential application in industry, agriculture, food or therapeutics. There are many advantages of using red kidney beans to study bioactive proteins. They are easy, fast to grow, and produce high numbers of seeds for isolation of cotyledons and embryos. They self-pollinate, also it is possible to get several generations under glasshouse conditions over the duration of this research. Moreover, the seeds are quite large, easy to handle, with plentiful proteins. It is easy to separate seed parts and prepare extracts.

Seeds are rich stores of protein, most of which are not essential in the germination process but might contribute to the defence of the seeds against different predators and pathogens (Sales *et al*., 2000). Defence proteins can protect organisms from stress or attack by foreign organisms. Different proteins with different bioactivities can play different roles in plant defence.
It has been reported that ecological and climatic factors may influence levels/concentrations of antifungal and antinutritional proteins in plants. For instance, lectins were detectable in the Indian velvet bean but when grown in Brazil, they were undetectable. A high level of trypsin inhibitor was detected in the velvet seeds in both countries (Siddhuraju *et al*., 1996; Udedibie and Carlini, 1998). It is possible that defence proteins in different cultivars may differ in sequences or bioactivities.

Although several antifungal proteins have been isolated from red kidney bean, French bean and Pinto bean seeds, firstly the antifungal proteins isolated from different varieties were not all identical in amino acid

sequence, type and bioactivities (Ye and Ng, 2002d). In other words, different antifungal proteins can be produced by different varieties of the same species (Ye and Ng, 2002a). Secondly, AFPs have been isolated from the whole seed and therefore their localization in different seed and seedling parts (during germination or in response to stress factors) is not known. Even though many different antifungal proteins have been isolated from different seeds, there is little information on the accumulation of these proteins during seed development and germination in red kidney bean. Thirdly, isolated proteins (like chitinase) have not been assessed for their ability to inhibit fungi and the bioactivities have just been reported. It is understandable that plant parts should be able to protect themselves from pathogen attack or abiotic stress. In this research bean seed tissues were screened for bioactivities following exposure to fungal metabolites or UV irradiation. Fourthly, sometimes in a single species a combination of antifungal proteins can be found (Dayler *et al*., 2005; Wang and Ng, 2003) and some proteins may also have overlapping biological activities. Moreover, bifunctionality has been reported in a single protein which can act against enzymes (Sancho *et al*., 2003). Thus it would be of interest to look for any bifunctional protein in red kidney bean seeds.

Sometimes some antifungal proteins may have synergistic effect together (e.g. defensins with thionins, Kirubakaran *et al*., 2008) that consequently will have a potent antifungal activity against pathogens or even presence of a multitude of antifungal proteins has been demonstrated (Wang and Ng, 2003). Among antifungal proteins, chitinases and β-1, 3- glucanases have

been the most attractive because of their strong activities against fungi *in vitro* (Muthukrishnan *et al*., 2001).

Our knowledge of the accumulation and changes in AFPs during seed development and germination is very limited because nearly all previous studies examined dry mature seeds only and few studies investigated their changes during germination (Petruzzelli *et al*., 2003; Muzquiz *et al*., 2004; Valdes-Rodriguez *et al*., 2007) and rarely during seed development from anthesis or early seedling growth (Kumar *et al*., 2005; Nielsen *et al*., 2006; Rodriguez-Herrera *et al*., 2006). Also, there is a paucity of information on the effects of biotic and abiotic factors on antifungal activity during germination of many seeds including red kidney bean seeds although work on sunflower seeds has shown some interesting results (Gonorazky *et al*., 2005). Therefore, there is a need for more comprehensive investigations in to the antifungal activity and characteristics of proteins in extracts from different seed tissues prepared from various stages of seed development to early seedling growth.

The specific objectives of this study were:

1. To investigate bioactive effects of crude extracts prepared from whole red kidney bean seeds and seedling parts including *in vitro* antifungal activity on *Alternaria alternata*. To purify AFP with activity against *A. alternata* and to determine its activity against amylases and proteases from different sources including those of *A. alternata*.

2. To determine the relative contribution of selected bioactive proteins including bovine trypsin, α-amylase inhibitory/stimulatory activities,

chitinase and β-1, 3- glucanase in seed and seedling parts to the observed *in vitro* growth inhibition of *A. alternata*.

3. To investigate other biological activities (such as conidiogenesis and melanization) of red kidney seed extracts on *A. alternata*.

4. To determine developmental changes of *in vitro* antifungal activity and four aforementioned activities in seed parts and pods during seed formation.

5. To investigate changes in the activities of the four proteins as well as *in vitro* antifungal activity of seed and seedling parts during seed germination under biotic and abiotic stress.

Chapter 2- General Materials and Methods

2.1- Seeds, fungi and chemicals

Seeds of red kidney beans (*Phaseolus vulgaris* L.) were purchased from a local health food store. The following fungi used in this study were subcultured from stock cultures maintained at -80^{0}C in the microbiology lab of the school of biological sciences, University of Canterbury.

Aspergillus niger

Alternaria alternata (this fungus was isolated from Rhododendrons and selected among all tested fungi for further research in this study)

Cladosporium fulvum

Fusarium culmorum

Fusarium oxysporum

Fusarium solani

Penicillium digitatum

Verticillium sp.

Also an isolate of *Alternaria radicina* was kindly provided by Lincoln University (Lincoln, New Zealand) for use in assay of antifungal activity of red kidney bean extract against it. All fungi were maintained on potato dextrose agar slants.

Some chemical companies have been stated individually elsewhere in the thesis and others are listed in Table 2.

Table 2.1- Chemical suppliers.

Chemical name and catalogue number	Name of supplier
Calcium chloride (Cat. 1007047), sodium hydroxide (Cat. 10252), ammonium sulphate (Cat. 100334C), sodium succinate (Cat. 30219), D (+)- glucose (Cat. 101174Y)	BDH, Poole, England
Potato dextrose agar (Cat. CM0139), agar (Cat. LP0013)	Oxoid, Hampshire, England
Potato dextrose broth (Cat. 0549-17-9)	Difco Laboratories, Detroit, USA
Millipore membrane (0.22 μm sterile filter units, Cat. No. SLGP033RS)	Millex-GP, Carrigtwohill Co., Cork, Ireland
Bovine serum albumin (Cat. 30063-572)	Invitrogen, Auckland, New Zeaand
Sodium acetate, potassium phosphate, sodium chloride	Smith BioLab, Palmerston North, New Zealand
All other chemicals including: Coomassie Brilliant Blue dye (Cat. B-0770), porcine pancreatic α-amylase (A3176, Type VI-B), *Aspergillus oryzae* α-amylase (Cat. A6211) dialysis tubing (Cat. D0530)	Sigma, St. Louis, Missouri, USA

All spectrophotometric assays were performed using a Bio-Rad spectrophotometer (SmartSpecPlus, Hercules, California, USA). All columns used in this study were purchased from Bio-Rad, (Hercules, California, USA).

2.2- Protein extraction

Seeds (35 g) were soaked in distilled water (100 ml) in a beaker for 15 h at room temperature and seed parts (cotyledons, embryonic axes and seed coat) were separated. The method of Le Berre-Anton *et al*. (1997) with some modifications was followed to extract heat stable and low-pH soluble proteins from the seed parts. Briefly, each type of seed part was homogenized separately with distilled water in a mortar and the homogenates were centrifuged at 6000 g for 10 min. The volume of supernatant was measured and then 0.11 ml of succinate buffer (pH 3.8) containing 100 mM $CaCl_2.2H_2O$ per ml of the supernatant was added.

The mixture was heated at 70^0C for 10 min and centrifuged at 10,000 g for 10 min. After this, the supernatant was recovered and its pH was adjusted to 5.6 by adding 0.1 M NaOH. The proteins that remained soluble were used in this study. Samples were freeze dried, dissolved and dialysed in an appropriate buffer for different experiments. This method was used in most parts of this research unless stated otherwise.

2.3- Water content

Water content of whole seeds, seed and seedling parts expressed as percentage and was calculated from the difference between their fresh weights and their dry weights after heating at 70^0C for 5 days (Ramos *et al*., 1998).

2.4- Protein content

The amounts of soluble proteins in different crude extracts or fractions eluted from different columns were quantified using the principle of

quantitative binding of proteins (Bradford, 1976) with the Coomassie Brilliant Blue dye. Bovine serum albumin was used as a standard.

2.5- Gel electrophoresis

Two different methods of PAGE were used. The chemicals used for non-denaturing-PAGE and SDS-PAGE were purchased from Bio-Rad (Hercules, California, USA). All other chemicals were purchased from Sigma (St. Louis, Missouri, USA).

2.5.1- SDS-PAGE

The progress of protein purification at different steps was assessed using the Laemmli's SDS-PAGE procedure (Laemmli 1970) on a Bio-Rad Mini Protean 3 slab gel apparatus. Gel preparation (12% resolving gel and 4% stacking gel) and staining with the Coomassie Brilliant Blue dye were according to the manual of BioRad Mini Protean 3 slab gel apparatus. When required, following SDS-PAGE, the gels were also silver-stained based on the method of Hoving *et al.* (2005). The Bio-Rad low range prestained SDS-PAGE standards were used consisting of the following proteins with their respective molecular masses given in brackets: phosphorylase B (103.035 KDa), bovine serum albumin (80.664 KDa), ovalbumin (49.491 KDa), carbonic anhydrase (36.545 KDa), soybean trypsin inhibitor (28.829 KDa) and lysozyme (19.445 KDa).

2.5.2- Native gel electrophoresis

To identify porcine pancreatic α-amylase and bovine trypsin inhibitor isoforms present in red kidney bean extracts, electrophoretic separation of proteins in the extracts on native polyacrylamide gels was first performed.

2.5.2.1- Detection of isoinhibitors of porcine pancreatic α-amylase in native gels

To detect α-amylase isoinhibitors in native polyacrylamide gels a method based on that of Giri and Kachole (1996) with minor modifications was used. Briefly, the protein extracts were mixed with sucrose (60%, w/v) to make a final 40% (w/v) sucrose solution and were separated using 7.5% non-denaturing polyacrylamide gels (pH 8.8) containing 0.5% (w/v) soluble potato starch and tris-glycine (pH 8.3) as the electrode buffer under a constant voltage (200v) for 39 min. After electrophoresis, the gel was incubated in 100 ml of 20 mM potassium phosphate buffer (pH 6.9) containing 6.7 mM NaCl for 10 min in a shaking water bath at 37^0C before transfer to the same buffer containing 0.5% (w/v) porcine pancreatic α-amylase for a further 10 min incubation in the water bath with gentle shaking. The gels were rinsed, placed in an iodine reagent (prepared from a stock solution consisting of 6% KI and 0.6% iodine in HCl 0.05 N) for one h, then put on a light box and photographed using a Nikon D700 camera with Nikon 60 mm macro lens. Preparation and electrophoresis conditions of the mini slab gels for non-denaturing PAGE was according to the manual of the Bio-Rad Mini Protean 3 gel apparatus.

2.5.2.2- Native gels for detection of bovine trypsin isoinhibitors in seed extracts

This method involving immobilization of azoalbumin in native polyacrylamide gels for detection of bovine trypsin isoinhibitors was developed in our lab for the first time. Trypsin isoinhibitors in seed extracts were separated under non-denaturing conditions on a Bio-Rad mini vertical slab gel electrophoresis system with 7.5% polyacrylamide containing 3%

(w/v) azoalbumin as substrate, 4% stacking gel and Tris-glycine (pH 8.3) as electrode buffer. Seed extracts were mixed with sucrose (60%, w/v) to make a final 40% (w/v) sucrose solution before loading to the wells of a gel. In preliminary experiments, 1 mg/ml of a soybean trypsin inhibitor (cat. T6522, Sigma) dissolved in 200 mM Tris-HCl buffer at pH 8 containing 0.2 M $CaCl_2.H_2O$ was used instead of seed extracts as a positive control. After electrophoresis at a constant voltage (200 V) for 39 min, the gel was washed and placed in 200 mM Tris buffer containing 0.2 M $CaCl_2.H_2O$ at pH 8 for 10 min at 37^0C for equilibration before transfer to the same buffer containing bovine trypsin (13.7 mg/100 ml in the same Tris buffer). This was incubated at 37^0C for 40 min. The gel was then washed briefly with distilled water, put on a light box and photographed using a Nikon D700 camera with Nikon 60 mm macro lens.

2.6- Assay of antifungal activity

Two different methods were employed for assessment of antifungal activity. To detect the presence of antifungal activity in seed extracts a paper disc method was used. For determination of change of antifungal activity during seed development, or in response to different stress treatments a method based on a constant amount of soluble seed protein mixed with the medium (incorporating of protein into agar plates) was applied.

2.6.1- Paper disc method

All extracts were screened for their antifungal activity according to a method described by Ye and Ng (2002a) using 80 × 12 mm Petri plates containing 10 ml of potato dextrose agar. A small disc from the growing margin of a 3-

day-old fungal colony on PDA was placed at centre of the plate and incubated at 26^0C for 72 h. Paper discs (7 mm diameter) were punched from Whatman filter paper (No. 3) and four discs were placed equidistant in each plate (Fig. 2.1). The discs were treated with crude extract containing 12 µg soluble proteins and the plates were incubated at 26^0C±1 for 24 h. The zones of inhibition around discs were considered as presence of antifungal protein and the distance between the disc and the colony margin was measured using a ruler and compared with control discs of either boiled protein (20 min) or papain degraded proteins. All fungal species listed in 2.1 were tested.

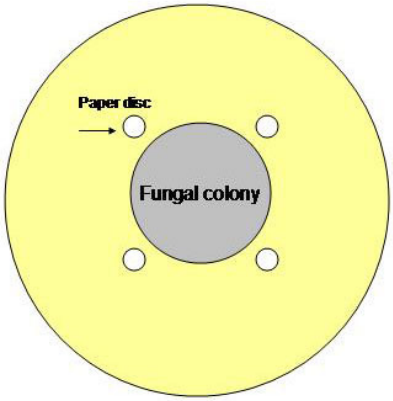

Figure 2.1- Paper disc method scheme.

2.6.2- Incorporation of protein into agar method

Assessment of changes in AFPs in seedling parts in response to stress treatments or during seed germination (0, 1, 2, 5, 7 and 11 days of germination at 26^0C) and development (20, 25, 30, 35, 40, 45 and 50 days after flower fall) was carried out using this method. Extracts each containing 20 µg soluble proteins from different parts of seeds or seedlings as well as root exudates were added to PDA (with the final volume equal to 4 ml) just

prior to agar setting (45^0C). A small disc from the growing margin of a 3-day-old *Alternaria alternata* (this fungus was selected for antifungal experiments) colony on PDA was placed at the centre of a plate and incubated at 26^0C for 72 h. Each Petri plate was photographed to record the areas of the whole plate and mycelia colony using a Photoshop software (Adobe Photoshop CS3, Version 10.0, Adobe System Incorporated, 2007, USA). The pixels of both whole Petri plate and colony were recorded and based on the proportion between pixels and area, the area of the colony was calculated. At first calibration of this method was made using Petri dishes with different sizes of circles painted on their centres. It was found that before recording the data it was important first to click for histogram with uncached data and then read pixels for each Petri dish. One unit of antifungal protein (AFPU) was defined as the amount of AFP which decreases 0.1 cm^2 of the area of a mycelia colony relative to control after incubation.

2.7- Assays of biochemical activities

Among different bioactivities in red kidney beans, the following biochemical activities were evaluated for their possible association with antifungal activity in red kidney bean extracts.

2.7.1- Chitinolytic activity assay

All extracts were tested for chitinolytic activity using either chitin azure (Sigma, Cat. C-3020, St. Louis, USA) or carboxy methyl-chitin-remazol brilliant violet (CM-chitin-RBV) (Loewe Biochemica GmbH, Cat. D-83246, Munchen, Germany) as a substrate. Red kidney bean extracts each containing 18 µg soluble proteins in 500 µl acetate buffer (100 mM pH 5.2)

was mixed with 200 µL of 0.3% chitin azure in the acetate buffer. This mixture was incubated for 1 h at 37^0C before the reaction was stopped by adding 500 µL 0.5 M HCl. Following incubation on ice for 5 min, the samples were centrifuged at 10000 g for 10 min and the increase in absorbance relative to the enzyme control was measured at 550 nm (Guo *et al*., 2004). For experiments with CM-chitin-RBV as a substrate the same method and buffer were used except 200 µl of CM-chitin-RBV instead of chitin azure (Sluyter *et al*., 2005). Each assay was carried out in triplicates and chitinase from *Streptomyces griseus* (Cat. C-6137, Sigma, St. Louis, Missouri, USA) was used as a positive control. One chitinase unit (1 U) was defined as the amount of enzyme that increased the absorbance at 550 nm by 0.1 absorbance unit during 1 h at 37^0C.

2.7.2- Activity assay for β-1, 3- glucanase

β-1, 3- Glucanase activity was assayed using carboxy methyl linked with the dye remazol brilliant blue (CM-Curdlan-RBB) (Loewe Biochemica GmbH, Munchen, Germany, Cat. D-82054) as a substrate. The assay mixture comprised 500 µL acetate buffer (100 mM pH 5.2), 85 µg seed extract and 200 µL substrate. This was incubated for 2 h at 37^0C before the reaction was stopped by adding 500 µL 0.5 M HCl. Following incubation on ice for 5 min, it was centrifuged at 10000 g for 10 min. The increase in absorbance relative to the enzyme control was measured at 600 nm (Hartl *et al*., 2011) and one β-1, 3- glucanase unit (1 U) was defined as the amount of enzyme that increased absorbance at 600 nm by 0.1 unit under the assay conditions.

2.7.3- Activity assay of α-amylase inhibitor

Two different methods with soluble potato starch as a substrate for assaying the activity of porcine pancreatic α-amylase (PPA), *Aspergillus oryzae* and *Alternaria alternata* amylases were used. Crude seed extract containing 1 µg soluble proteins was used as PPA inhibitor.

2.7.3.1- Starch-iodine complex method

In the first method starch-iodine complex was used to detect amylase inhibitory activity following a method described before (Moreno *et al*., 1994) with some modifications. Briefly, 5 µl of PPA (4 mg dissolved in 1 ml of 100 mM sodium acetate buffer, pH 5.6) was pre-incubated with 195 µl of bean protein extract each containing 1µg soluble proteins for 10 min at 37^0C. Then 300 µl of soluble potato starch (0.1%, w/v, in the same buffer) was added. This was incubated at the same temperature for 5 min before the reaction mixture was stopped with the addition of 0.5 ml of an iodine reagent. The absorbance of all reaction mixtures was read at 620 nm. In the enzyme control tubes, acetate buffer instead of PPA was added with or without the bean seed extracts. One α-amylase inhibitor unit (1 UI) was defined as the amount of inhibitor that prevented a decrease of 0.1 absorbance unit at 620 nm during 5 min of the assay. One amylase unit (AU) was also expressed as the amount of the enzyme that gave a reduction by 0.1 absorbance unit relative to amylase control reactions during the assay.

2.7.3.2- Reducing sugar assay method

All chemicals in this part including 3,5- dinitrosalicylic acid (Cat. 28235), sodium sulfite (Cat. 10264) and potassium sodium tartrate (Cat. 102194Q)

were purchased from BDH, Poole, England. In the second method, reducing sugar release as a result of starch degradation was determined using a previously described method (Wang *et al*., 1997). Briefly, the assay procedure was the same as in 2.7.3.1 until the end of 5 min of incubation when 0.5 ml of DNS reagent (44 mM 3,5- dinitrosalicylic acid, 4 mM sodium sulfite and 375 mM sodium hydroxide), 0.5 ml of 1.4 M Rochelle salt (potassium sodium tartrate) and 0.5 ml deionized water were added to each of the reaction mixtures while standing on ice. Then these were heated in a boiling water bath for 10 min. After cooling to room temperature, absorbance at 575 nm was recorded. Different concentrations of glucose were used as reducing sugar standards. One inhibitor or stimulator unit was defined as the amount of inhibitor or stimulator that gave a reduction or increase by 0.1 absorbance unit at 575 nm relative to amylase control reactions during 5 min of the assay, respectively.

2.7.4- Assay of trypsin inhibitory activity

Extracts of seeds or seedling parts were tested for trypsin (EC 3.4.21.4) inhibitory activity against bovine trypsin (Cat. T-8003, Sigma) or protease extracted from *Alternaria alternata*. This assay was carried out with crude seed extracts (containing 10 µg soluble proteins) and using 1% (w/v) azoalbumin (Cat. A-2382, Sigma) dissolved in distilled water as substrate following a previously described method with some modifications (Sarath *et al*., 1989). Briefly, trypsin (25 µg in 100 µl 200 mM Tris-HCl buffer at pH 8 containing 0.2 M $CaCl_2.H_2O$) and seeds or seedling extract (10 µg protein in 100 µl of the same buffer) were incubated for 8 min at 37^0C. Then 300 µl of substrate was added and reaction was allowed to proceed for 25 min at the

same temperature before it was terminated with addition of one ml of trichloroacetic acid (5%, w/v) (Cat. BSPTA321, Biolab, Australia). The difference in absorbance due to breakdown products at 450 nm between trypsin reaction with the substrate in the presence or absence of a seed extract was determined after centrifugation of the reaction mixtures at 10,000g for 10 min. One trypsin inhibitor unit (TIU) was defined as the amount of inhibitor that reduced absorbance at 450 nm by 0.1 unit relative to trypsin control reactions for 25 min. One protease unit (PU) was also expressed as the amount of enzyme that increased absorbance by 0.1 unit at 450 nm.

2.8- Statistical analysis

All experiments were repeated for at least two times and all treatments were carried out at least in three replicates. Data were subjected to one-way analysis of variance (ANOVA, $p \leq 0.05$) followed by comparison of mean values of the treatments using Duncan's Multiple Range test at 5% level of significance (Clewer and Scarisbrick 2001).

Wherever data transformation was required they were transformed and then analysed.

Chapter 3- Studies on Selected Bioactive Proteins from Mature Red Kidney Bean Seeds

3.1- Introduction

Leguminous plants have been a subject of research due to their possession of a huge amount of proteins and peptides with important biological activities (Wang *et al*., 2009a). To date several antifungal proteins have been reported from different cultivars of *Phaseolus vulgaris* (Table 3.1). Their antifungal activities have been investigated under *in vitro* conditions. For example, a thaumatin-like protein (molecular mass of 20 KDa) isolated from French beans (*Phaseolus vulgaris* cv. Kentucky Wonder) was shown to have antifungal activity against *Fusarium oxysporum*, *Coprinus comatus* and *Pleurotus ostreatus* but not *Rhizoctonia solani* (Ye *et al*., 1999). From dried flageolet beans (*Phaseolus vulgaris* cv. Flageolet Bean) an antifungal protein (molecular mass of 33 KDa) with activity against *Mycosphaerella arachidicola* but not *F. oxysporum* and *Botrytis cinerea* was purified (Xia and Ng, 2005). The protein also showed hemagglutinating activity.

Table 3.1- Different antifungal proteins and peptides isolated from different cultivars of *Phaseolus vulgaris*. Abbreviations of fungal scientific names: *B.c = Botrytis cinerea*; *C.c = Coprinus comatus*; *F.l = Fusarium lateritum*; *F.o = Fusarium oxysporum*; *F.s = Fusarium solani*; *H.m = Helminthosporium maydis*; *M.a = Mycosphaerella arachidicola*; *P.a = Pythium aphanidermatum*; *P.o = Pleurotus ostreatus*; *P.p = Physalospora piricola*; *R.s = Rhizoctonia solani*; *V.m = Valsa mali*. Abbreviations of bacterial scientific names: *B.m = Bacillus megaterium*; *B.s = Bacillus subtilis*; *M.p = Mycobacterium phlei*; *P.v = Proteum vulgaris*.

Cultivar name	**Isolated protein/peptide**	**Antifungal activity**	**Molecular weight**	**Reference**
Kentucky Wonder (French bean)	A thaumatin-like protein	*C.c*, *F.o*, *P.o*, but not *R.s*	20 KDa	Ye *et al*., 1999
Red kidney bean	A homodimeric lectin	*C.c*, *F.o*, *R.s*	67 KDa	Ye *et al*., 2001b
Pinto bean	An antifungal peptide	*B.c*, *F.o*, *M.a*,	5 KDa	Ye and Ng, 2001b
Kentucky Wonder (French bean)	A peroxidase	*B.c*, *C.c*, *F.o*, *M.a*	37 KDa	Ye and Ng, 2002a
Pinto bean	A chitinase A novel antifungal protein	*F.o*, *P.p*, *R.s* Different potencies in the proteins was observed	28 KDa 32 KDa	Ye and Ng, 2002d
Pinto bean	An antifungal polypeptide	*B.c*, *C.c*, *F.o*, *M.a*	28 KDa	Ye and Ng, 2003
Flageolet bean	An antifungal protein with hemagglutinating activity	*M.a* but not *B.c*, *F.o*	33 KDa	Xia and Ng, 2005
Black Turtle	A chitinase-like antifungal protein	*F.o*, *M.a*	28 KDa	Chu and Ng, 2005

Haricot bean	An antifungal & antibacterial peptide (Vulgarinin)	*B.c*, *F.o*, *M.a*, *P.p* bacteria: *B.m*, *B.s*, *M.p*, *P.v*	7 KDa	Wong and Ng, 2005a
Spotted Bean	An antifungal peptide	*F.o*, *M.a*	7.3 KDa	Wang and Ng, 2007b
Perola	An antifungal defensin	*F.l*, *F.o*, *F.s*, *R.s*	6 KDa	Games *et al*., 2008
French Bean	A defensin-like antifungal peptide	*M.a*, *R.s*, *V.m*	6 KDa	Leung *et al*., 2008
Canadian cranberry beans	A chitinase	*B.c*, *F.o*, *P.a*, *P.p*	30.6 KDa	Wang *et al*., 2009b
French bean number 35	An antifungal protein with hemagglutinating activity	*V.m* but not *F.o*, *H.m*, *M.a*, *R.s*	64 KDa	Lam and Ng, 2010

No antifungal activity was observed in a number of hemagglutinins and lectins isolated from common bean cultivars (Xia and Ng, 2006; Leung *et al*., 2008; Sharma *et al*., 2009). All antifungal proteins which have been isolated from different varieties of *Phaseolus vulgaris* including red kidney beans, pinto beans and French beans are not identical in type, bioactivities and amino acid sequences (Ye and Ng, 2002d). None of those antifungal proteins isolated from common bean have been studied for either their enzymatic activities against fungi or their inhibitory activities against hydrolytic enzymes. The main focus of these works has been placed on identification and isolation of the proteins and their activity against *in vitro* growth of a few selected fungi. However, the fungicidal or fungistatic effects

of these proteins, their mode of action or their effect on spore germination are largely unknown.

Common bean seeds contain anti-nutritional proteins including trypsin and α-amylase inhibitors (Le Berre-Anton *et al*., 1997; Piergiovanni and Pignone, 2003; Kluh *et al*., 2005; Guillamon *et al*., 2008; Alves *et al*., 2010). Interestingly trypsin inhibitors from other seeds have also been shown to possess antifungal activity. However, there is no report on whether the trypsin inhibitor of the common bean could also inhibit fungal growth or protease activity of fungi (Dunaevskii *et al*., 1997; Chilosi *et al*., 2000; Wang and Ng, 2006b; Yang *et al*., 2006). α-Amylase inhibitors, other well known antinutritional proteins from the common bean, have been assessed against amylases from different sources including insect, nematode and two phytopathogenic fungi. Insect amylases were generally inhibited by these proteins while there was weaker activity against nematode amylase and no inhibition against amylases of *Fusarium* spp. and *Sclerotinia* spp. (Grossi de Sa *et al*., 1997; Kluh *et al*., 2005). Enzymes that hydrolyze the fungal cell wall such as chitinases and β-1,3-glucanases also help seeds to defend themselves against pathogens (Gomez *et al*., 2002; Leubner-Metzger 2003). Chitinase activity was detected in cotyledons, embryonic axis and seed coat of two different cultivars (Maisugata and Surattowonder) of *Phaseolus vulgaris* but the connection of this with antifungal activity was not assessed (Ramos *et al*., 1998). Interestingly, a thermostable chitinase with antifungal activity has recently been isolated from Canadian cranberry beans (*Phaseolus vulgaris*). It inhibited growth of *F. oxysporum*, *Physalospora piricola*, *Botrytis cinerea* and *Pythium aphanidermatum* (Wang *et al*., 2009b). It has been reported that the β-1,3-glucanase of cowpea seeds

(*Vigna unguiculate* L Walp) showed antifungal activity against *Colletotrichum musae* (an agent of banana peel anthracnose) and *C. lindemuthianum* (an agent of anthracnose on many plants) (Gomes *et al*., 1996). β-1,3-Glucanase has been isolated and purified from bean leaves (Abeles and Forrence, 1970; Abeles *et al*., 1970) but there is no report regarding occurrence of β-1,3-glucanase in the common bean seed.

The main objective of research described in this chapter was to purify an antifungal protein from red kidney bean seed against *Alternaria alternata* and then determine some of its properties. In particular, an assessment was made to see whether trypsin, α-amylase inhibitors, chitinases and β-1,3-glucanases which were also present in the red kidney bean seed extract might be involved in the observed antifungal activity or not.

While working on the project it was found that the fungus accumulated melanin in response to red kidney bean crude extracts. There was no report about the effect of proteins on melanization. Therefore, effect of crude protein extracts of red kidney bean seed on melanization of *A. alternata* as well as their inhibitory activity against amylase and protease activities of the fungus were also investigated. In addition, the AFP (against *A. alternata*) after purification from red kidney bean seed extracts was investigated to determine if it had chitinase, β-1,3-glucanase, amylase and trypsin inhibitory activities. Moreover, its antifungal activity was investigated more closely regarding whether it was fungicidal or fungistatic as well as its effect on the morphological changes of the fungus during mycelia growth and spore germination.

3.2- Materials and methods

The strain of *Alternaria alternata* was obtained from the microbiology lab of the school of biological sciences, University of Canterbury. It had originally been isolated from a Rhododendron plant.

3.2.1- Extraction methods

Proteins were extracted from both dry red kidney bean seeds (to assess bioactive effects) and *A. alternata* (as a source of fungal amylase and protease).

3.2.1.1- Seed protein extraction

The method described in 2.2 was used except that seeds were not separated into seed parts. The seed extracts obtained were used in the experiments described in this chapter.

3.2.1.2- Fungus culture and protein extraction

Extracts prepared from culture of an isolate of *A. alternata* was tested for amylase and protease activities. These extracts were also used to detect if red kidney bean extracts had inhibition against these fungal enzymes.

3.2.1.2.1- Fungal protein extraction for amylolytic assay

An inoculum consisting of a 5 mm diameter PDA plug excised from the margin of a 3-day-old fungus culture was transferred and cultured at 26^0C for 4 days in 50 ml sterile liquid medium (0.025 g $MgSO_4.7H_2O$ (Cat. 101514Y, BDH), 1.5 g K_2HPO_4, 0.025 g $CaCl_2$, 0.015 g $FeSO_4.7\ H_2O$ and

0.005 $ZnSO_4.7H_2O$ per litre distilled water, pH 6.2, Upadhyay *et al*., 2005) containing 50 g potato starch in 250 ml flasks on an orbital shaker (200 rpm). The culture was then centrifuged at 10,000g and 4^0C for 10 min. The supernatant obtained was filtered through Whatman filter paper (No. 1) and the filtrate was considered as extracellular proteins of the culture. The precipitated fungus was washed with distilled water and ground biomass in a mortar to powder with the aid of liquid nitrogen. Then acetate buffer (100 mM, pH 5.6) was added to the powder (1:5 w/v) yielding a homogenate which was centrifuged at 10,000g and 4^0C for 10 min. The supernatant thus obtained can be referred to as a fungal intracellular protein extract (Guimaraes *et al*., 2006). Both intracellular and extracellular extracts, were mixed with ammonium sulphate (85% saturation) and centrifuged at 4^0C at 10,000 g for 10 min (Kamath *et al*., 2010). Pellets obtained after centrifugation, were resuspended in acetate buffer (100 mM acetate buffer at pH 5.6) and later checked for amylolytic activity. Medium without inoculum was used as control.

3.2.1.2.2- Fungal protein extraction for protease assay

Wheat bran Czapek Dox medium was used for culturing *A. alternata* in an experiment to study protease production by the fungus using a method modified from that of Patil and Shastri (1985). The fungus was cultured in 250 ml flasks containing 50 ml medium (15 g sucrose, 15 g fructose), 20 g wheat bran, 2 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 0.01 g $FeSO_4.7\ H_2O$ and 10 g casein (Cat. 440164R, BDH) per litre distilled water at pH 6.2) and incubated at 26^0C on an orbital shaker (200 rpm) for 4 days. Casein was sterilized separately by a modified tyndallisation method

(heating the casein solution in a boiling water bath for 15 min for three consecutive days) as described before (Aminot and Keroul, 1997) and later it was mixed with the rest of the medium ingredients. The procedure to obtain intra- and extracellular extracts from fungal culture for protease activity assay was described in 3.2.1.2.1.

3.2.2- Assay of fungal amylase and protease activity

3.2.2.1- Assay of fungal amylase activity

Production of amylase in *A. alternata* was confirmed using two different methods.

3.2.2.1.1- Agar-plate method

Starch hydrolysis by a growing fungal mycelium was detected on a medium containing ingredients mentioned in 3.2.1.2.1 and 16 g agar (Cat. LP0013, Oxoid, Hampshire, England) per litre distilled water. The medium was autoclaved and poured into Petri plates (10 ml of medium per plate). An inoculum consisting of some hypha excised from a 3-day-old fungus cultured on PDA medium was placed on the centre of the medium and then incubated at 26^0C in the dark for 4 days. After this, the surface of the Petri plates was covered with an iodine solution (its composition and preparation as described in 2.5.2.1) for 30 s before the excess reagent was poured off. Appearance a clear zone free of blue iodine-starch staining in the medium surrounding the fungal mycelium was taken as a positive reaction of starch hydrolysis (Aneja, 2005).

3.2.2.1.2- In-solution (test tube) method

Amylase activity of extra- and intracellular fungal proteins were also determined using a method as described in 2.7.3.1 and 2.7.3.2 except that the incubation time was 1 h and incubation temperature was 40^0C in this experiment. Each extract was diluted with acetate buffer to contain 10 µg soluble proteins.

3.2.2.2- Assay of fungal protease production

Presence or absence of proteolytic activity in a culture of *A. alternata* was confirmed using two different methods.

3.2.2.2.1- In-Petri dish (medium) method

Proteolytic activity in a fungal extract was detected on a medium following a method described by Ong and Gaucher, 1973. Briefly, a medium containing 4 g casein (Cat. 440164R, BDH), 0.4 g glucose, 0.3 g yeast (Cat. 11929, BBL, Cockcysville, USA), 0.01 g $FeSO_4.7\ H_2O$, 0.05 g KCl, 0.05 g $MgSO_4.7H_2O$, 0.1 g K_2HPO_4, 0.2 g $NaNO_3$ and 1.3 g agar per 100 ml distilled water was prepared and pH was adjusted to 6.2. The casein solution was sterilized separately as described in 3.2.1.2.2 and the rest of medium ingredients were autoclaved, later mixed with sterilized casein before pouring into Petri plates (10 ml of medium per plate). An inoculum consisting of 3-day-old fungus cultured on PDA medium was placed on the centre of the medium and was then incubated at 26^0C in the dark for 4 days. After this, the surface of the Petri plates was covered with aqueous 10%

(w/v) $HgCl_2$ and appearance of a clear zone in the medium surrounding the fungal mycelium was taken as a positive reaction of protein hydrolysis.

3.2.2.2.2- In-solution (test tube) method

Proteolytic activity of extra- and intracellular fungal proteins was also determined using a method described in 2.7.4 except that the incubation time was 2 h and also the buffer in this experiment was acetate buffer (100 mM pH 5.6). Each extract was diluted with the acetate buffer to contain 20 µg soluble proteins.

3.2.3- Bioactivities of extracts of whole red kidney bean seed

3.2.3.1- Assays of chitinolytic and β-1, 3- glucanase activities in crude seed extracts

Assays of chitinolytic and β-1, 3- glucanase activities in crude seed extracts were carried out as described in 2.7.1 and 2.7.2.

3.2.3.2- Assays of amylase and protease inhibitors

Inhibitory activity against porcine pancreatic α-amylase and bovine trypsin in crude seed extracts was determined as described in 2.7.3.1 and 2.7.4.

Each extract was diluted with acetate buffer to contain 1 µg soluble proteins in assays with porcine pancreatic α-amylase or 30 µg with amylases of *A. alternata* and *Aspergillus oryzae*. For bovine trypsin inhibitory activity assays, each extract was diluted with tris-HCL buffer to contain 10 µg soluble proteins or diluted with acetate buffer to contain 4 µg soluble proteins in assays with *A. alternata* protease.

3.2.3.3- Antifungal activity *in vitro*

The presence of antifungal activity in crude seed extracts against the fungi listed in 2.1 was detected using a paper disc method described on 2.6.1.

3.2.3.4- Assay of melanin induction in submerged culture of *Alternaria alternata*

Melanin content in the fungus induced by red kidney bean crude extract was compared with control as described below.

3.2.3.4.1- Fungal culture and melanin induction test

An inoculum consisting of some hyphae excised from a 3-day-old *A. alternata* grown on PDA medium was cultured in sterile 250 ml jars containing 50 ml potato dextrose broth (24 g/l) and incubated at 26^0C in the dark on an orbital shaker (200 rpm) for 2 days before crude red kidney bean seed extract containing 100 µg protein in 2 ml of 10 mM tris-HCl buffer at pH 7.3 was added to each culture jar under aseptic condition in a laminar flow cabinet. As a control, 2 ml tris-HCl buffer without crude seed extract was added to another culture jar. The crude seed extracts were filter-sterilized using Millipore express membrane (0.22 µm sterile filter units, Cat. No. SLGP033RS, Millex-GP, Carrigtwohill Co., Cork, Ireland) before use. The cultures were then incubated for another day before they were centrifuged at 10,000 g for 10 min. The fungal biomass was harvested and washed with distilled water before melanin extraction (Babitskaya *et al*., 2000). Mycelia were mixed with 2% NaOH (1:10 w/v) at 100^0C for 2 h in a boiling water bath. The pH of the extract was adjusted to 2 using

concentrated HCl when it was cooled down to room temperature. After centrifugation at 6,000g for 10 min, the pellet containing coagulated pigment was separated and dissolved in 5 ml 2% NaOH (w/v) before absorbance was read at 459 nm.

3.2.4- Antifungal protein studies

To study the antifungal protein against *Alternaria alternata*, the protein was first purified and then its biochemical properties were assessed.

3.2.4.1- Purification of antifungal protein (AFP) against *Alternaria alternata*

Antifungal protein from red kidney bean seeds was purified using different column chromatographic procedures. At each stage of purification, different fractions collected from columns were assessed for antifungal activity (2.6.1) against *A. alternata* and the extent of purification on SDS-PAGE as described in 2.5.1.

3.2.4.1.1- Protein extraction and preparation

Cotyledons and embryonic axes were excised from dry red kidney bean seeds and extracted using a method described in 2.2.

3.2.4.1.2- Protein purification

A method based on that of Xia and Ng (2005) with some modifications was used. The extracts obtained from 150 g seeds was fractionated as described in 3.2.4.1.1 with ammonium sulphate and the fraction obtained between 0-

85% saturation was dialysed (dialysis tubing, Cat. D0530, Sigma, St. Louis, Missouri, USA) against 20 mM phosphate buffer at pH 6.8. The dialyzed extract was subsequently applied to an anionic exchange column (30 × 1.5 cm) of DEAE cellulose (Cat. D-6418, Sigma, St. Louis, Missouri, USA) equilibrated with phosphate buffer. All unadsorbed proteins were eluted using the same buffer (100 ml). The bound proteins were desorbed following addition of 1 M NaCl in this buffer. The unadsorbed fraction from DEAE cellulose column was freeze dried, dialysed against 20 mM tris-HCl buffer pH 7.3 and applied to an affinity column (20 × 2.5 cm) of Affi-gel blue gel (Cat. 153-7302, Bio-Rad, Hercules, California, USA) which had been regenerated using 2 M Guanidine HCl (Cat. G4505, Sigma, St. Louis, Missouri, USA) in phosphate buffer according to the manufacturer's manual before equilibration with tris-HCl buffer. After removal of unadsorbed materials, retained proteins were displaced with the same buffer containing 1 M NaCl. The fraction of bound proteins was freeze dried and then dialysed against 10 mM NH_4OAc buffer at pH 4.6 before it was applied to a column (30 × 1.5 cm) of CM-Sepharose (CCF100, Sigma, St. Louis, Missouri, USA) which was equilibrated with the same buffer. After elution of unadsorbed proteins, the AFP was eluted using the aforementioned buffer containing 0.2 M NaCl. This fraction of bound proteins was desalted following dialysis against 20mM tris-HCl buffer at pH 7.3, freeze dried and stored at -20^0C until use in later experiments (Fig. 3.1). All column chromatography steps were carried out at 4^0C. Absorbance of all fractions collected was read at 280 with a spectrophotometer.

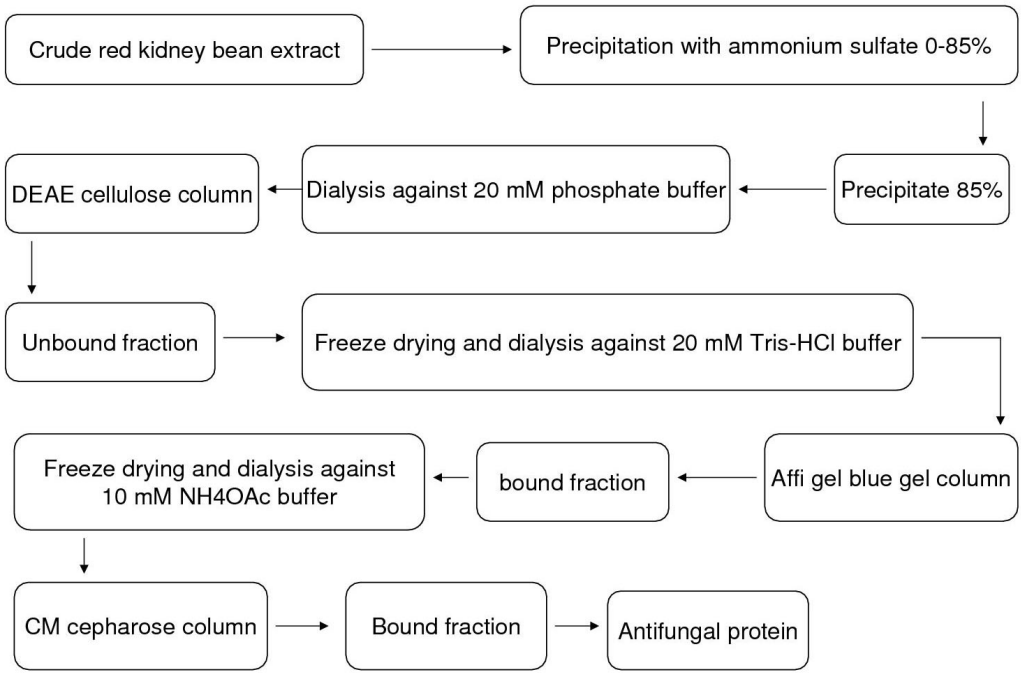

Figure 3.1- Flow-chart showing antifungal protein purification process from crude red kidney bean extract.

3.2.4.2- Electrophoresis and identification of the protein

A single band of the purified antifungal protein following SDS-PAGE (2.5.1) and staining with the Coomassie Brilliant Blue dye was excised and sent to the Australian Proteome Analysis Facility (APAF), Macquarie University, Sydney, Australia for N-terminal sequencing (for more details see the Appendices). The sequence data were searched against the UniProt amino acid sequence database.

3.2.4.3- Assay of the fungicidal and fungistatic effect of AFP

The purified AFP was assayed for any fungicidal or fungistatic effect basically as described by Theis *et al*. (2005) with some modifications. Conidial suspensions collected from 10-day-old culture of *A. alternata* grown on PDA by adding 10 ml of sterile YPG [0.3% yeast extract (Cat. 11929, BBL, Maryland, USA), 1% peptone and 2% glucose (Cat. 101174y, BDH, Poole, England) at pH 4.5] to the surface of the mycelium in a laminar flow cabinet. Conidia in the suspensions were counted using a hemacytometer (Ref. 0610030, Marienfeld, Lauda-Konigshoten, Germany) under a light microscope (Olympus Optical Co., LTD, Model CHK, Taiwan). After adjustment to an appropriate concentration, 5 ml medium containing 16×10^4 conidia of the fungus (*A. alternata*) and 200 µg purified AFP were incubated in sterile small centrifuge tubes (15 ml) at 26^0C in the dark for 12 h. The tubes were then centrifuged at 10,000 g for 10 min and the supernatant was discarded under aseptic conditions in a laminar flow cabinet. The harvested conidia were washed three times with sterile YPG. After this, 5 ml of sterile YPG was added to the washed conidia and were incubated for 48 h under the same conditions. For control, the medium without AFP was used. Delayed germination of conidia after removal of AFP was taken as fungistatic effect whereas no germination and growth after removal of AFP was taken as fungicidal activity.

3.2.4.4- Study on morphological changes induced in the fungus by red kidney bean AFP

This assay was carried out according to Singh *et al*., 2008 with some modifications. Briefly, an inoculum consisting of some hyphae excised from a 3-day-old *A. alternata* grown on PDA medium was placed in sterile Erlenmeyer flasks (100 ml) containing 10 ml potato dextrose broth and was incubated at 26^0C in a dark growth room with continuous shaking (200 rpm) for 2 days. After incubation, 100 µg filtered-sterilized AFP in 1 ml 20 mM phosphate buffer pH 6.8 was added to each flask under aseptic condition in a laminar flow cabinet (for control AFP was not added to the buffer). After returning to the dark growth room for another day, the cultures were examined under a light microscope (× 400) to observe any morphological changes.

3.2.4.5- Spore germination assay

This assay was carried out as previously described (Abad *et al*., 1996). Fifty microliters of 2 × PDB containing 23×10^4 conidia/ml (*A. alternata*) were mixed with 50 µl water, BSA or different concentrations of AFP in sterile Eppendorf tubes. The final treatments consisted of 11.5×10^4 conidia/ml which were incubated with 10, 15 or 20 µg/ml AFP or 50 µg/ml BSA (control) for 24 h at 26^0C. After incubation, the total numbers of ungerminated and germinated spores were counted using a hemacytometer. Data on the percentage germination of spores in comparison with the control were subjected to Arcsine square root transformation before one-way analysis of variance was carried out.

3.2.4.6- Assay of bioactive properties of the purified AFP

Assays of chitinolytic and β-1, 3- glucanase activities of purified antifungal protein were carried out as described in 2.7.1 and 2.7.2. The protein was also used for assays of inhibitory activities against porcine pancreatic α-amylase, bovine trypsin as well as *Alternaria alternata* protease as described in 2.7.3.1, 2.7.4 and 3.2.2.2.2.

3.3- Results

3.3.1- Amylase and protease production by *Alternaria alternata*

A clear zone appeared around the colony of *A. alternata* growing on starch- or casein-containing agar plate indicating production of amylase and protease by the fungus (Figs. 3.2B and 3.3B). This was also confirmed using in-solution amylase or protease assays (Figs. 3.2A and 3.3A). Both extracellular and intracellular extracts (each containing 10 or 20 μg protein of the fungus for amylase or protease assay, respectively) showed amylolytic and proteolytic activity. Higher levels of amylase and protease activities were found in the extracellular extracts ($P<0.05\%$).

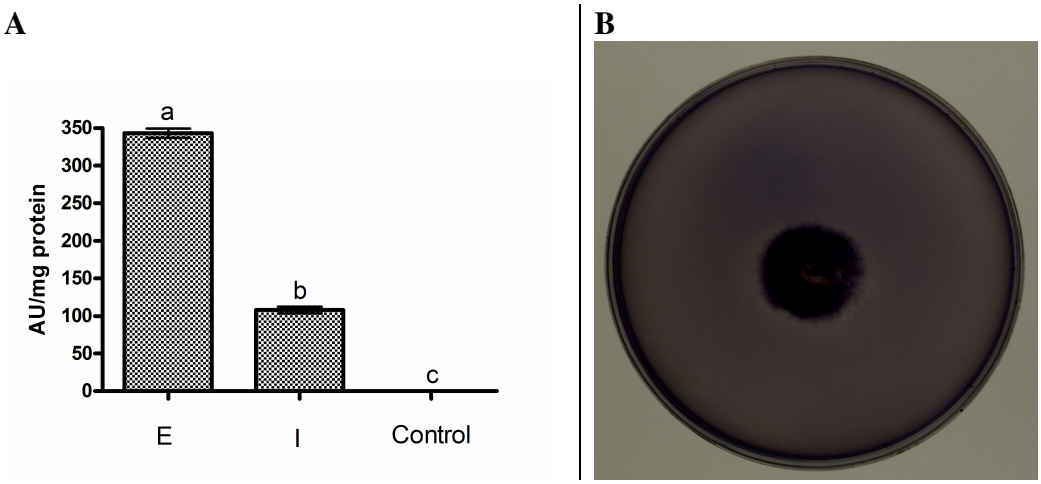

Figure 3.2- Amylase activity of *Alternaria alternata*. **A-** Amylase activity in intracellular (**I**) and extracellular (**E**) extracts of *Alternaria alternata*. Mean absorbance values labelled with different letters differ significantly according to Duncan's multiple range test (P<0.05). Control without addition of E or I. **B-** Appearance of a clear zone around *Alternaria alternata* colony due to starch hydrolysis.

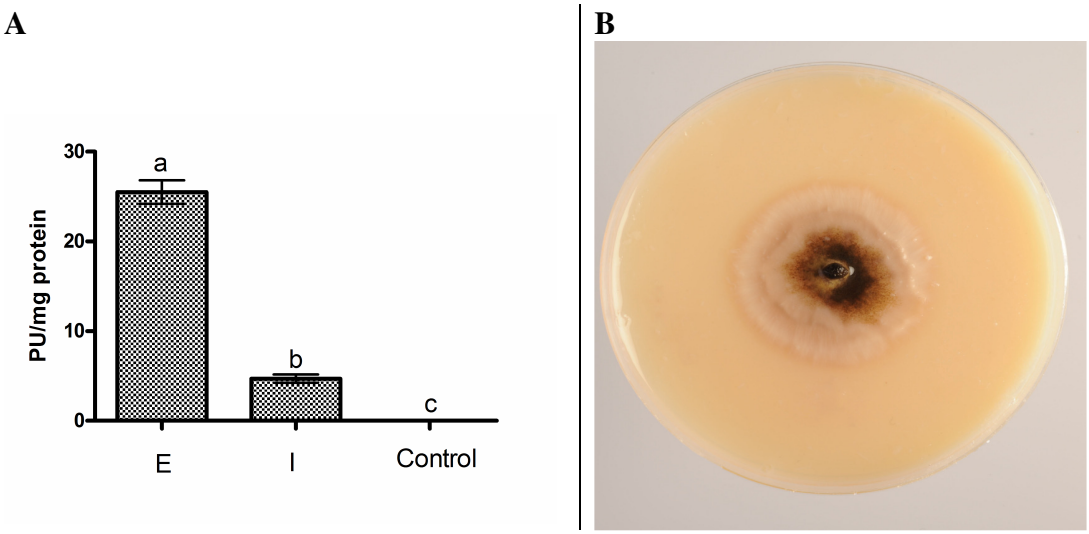

Figure 3.3- Protease activity of *Alternaria alternata*. **A-** Protease activity in intracellular (**I**) and extracellular (**E**) extracts of *Alternaria alternata*. Mean absorbance values labelled with different letters differ significantly according to Duncan's multiple range test (P<0.05). Control without addition of E or I. **B-** Appearance of a clear zone around *Alternaria alternata* colony showing protein hydrolysis.

3.3.2- Bioactive properties of crude seed extracts

3.3.2.1- Chitinolytic and β-1, 3- glucanase activities

Extracts of whole red kidney bean seeds showed chitinolytic activity compared to control without the extracts. The bean chitinolytic activity was significantly lower than that of purified chitinase from *Streptomyces griseus* which was used as a positive control (Figs. 3.4A and B). β-1, 3- Glucanase activity was also detected in the extracts of red kidney bean seed (Fig. 3.5A and B).

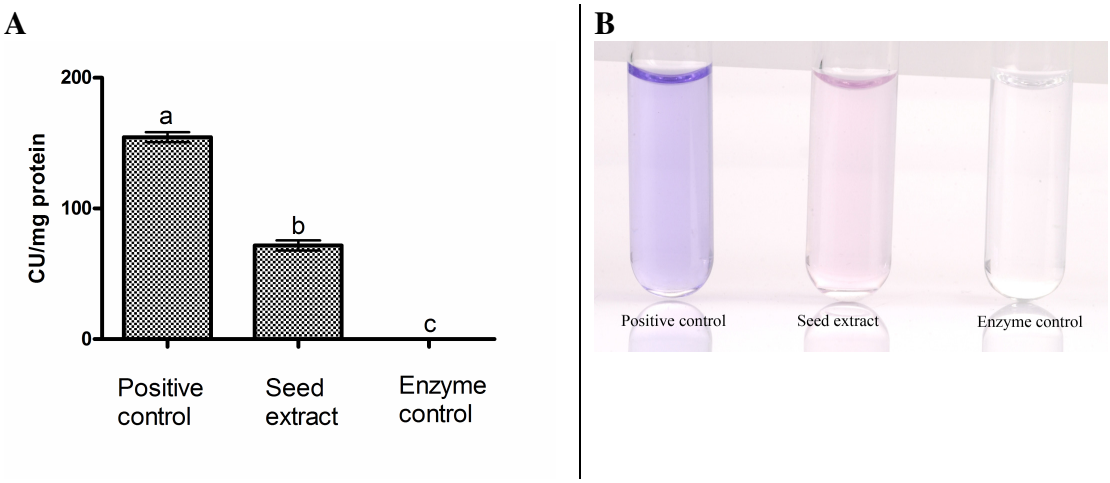

Figure 3.4- Chitinolytic activity in an extract of red kidney bean seed containing 18 µg soluble proteins, positive control was a chitinase from *Streptomyces griseus* and enzyme control was reaction in the absence of any added seed extract or enzyme (**A**). Values labelled with different letters differ significantly according to Duncan's multiple range test ($P<0.05$). **B-** Visual comparison of chitinase activity at the end of enzyme reaction in test tubes.

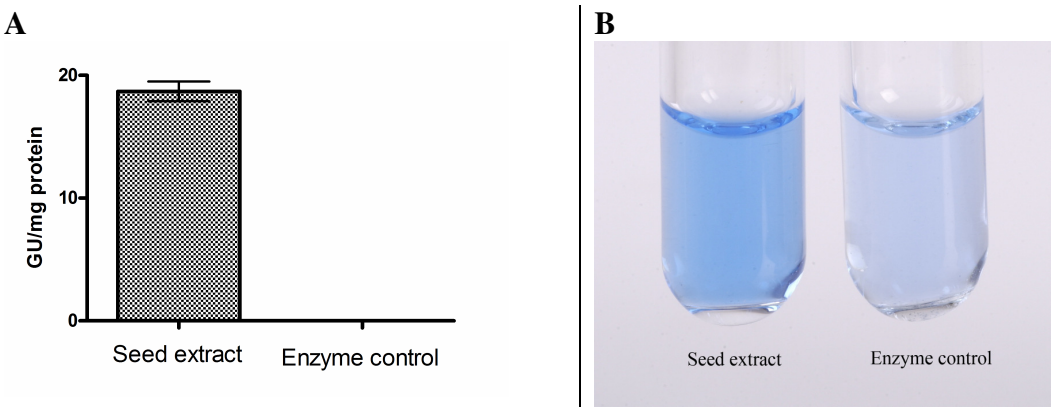

Figure 3.5- β-1, 3- Glucanase activity in an extract of red kidney bean seed containing 85 µg soluble proteins and enzyme control (**A**). **B**- Visual comparison of β-1, 3- glucanase activity at the end of enzyme reaction in test tubes.

3.3.2.2- Amylase and protease inhibitory activities in crude seed extracts

3.3.2.2.1- Can red kidney bean seed extracts inhibit amylase and protease activities of *Alternaria alternata*?

Soluble proteins (30 µg) from red kidney bean seeds were not able to inhibit the fungal amylase activity as no significant difference was observed between the fungal enzyme activities in the presence or absence of red kidney bean seed extracts (Fig. 3.6). Interestingly soluble proteins extracted from red kidney bean seeds were able to inhibit the fungal protease activity (Fig. 3.7). Extracts of red kidney bean seeds inhibited about 85% of the fungal protease activity. When bovine serum albumin (BSA) was used as a protein control, no significant difference was observed in the fungal protease activity in the presence or absence of BSA.

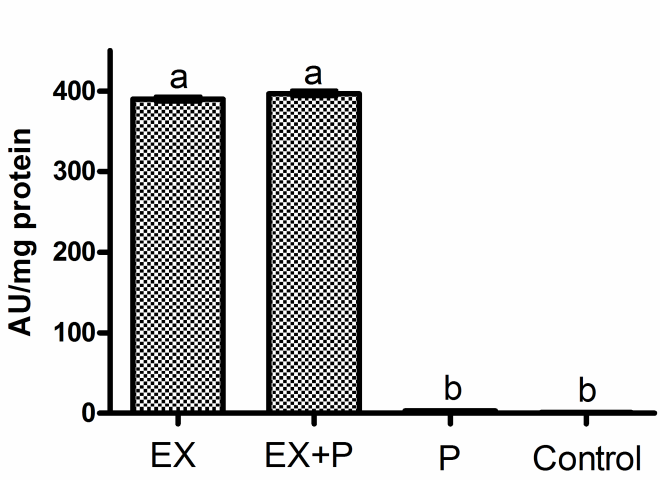

Figure 3.6- Assay of inhibitory activity of red kidney bean seed extracts containing 30 µg soluble proteins against extracellular amylase of *Alternaria alternata*. **EX**= Extracellular proteins of the fungus, **P**= Seed soluble proteins. Values labelled with the same letter do not differ significantly according to Duncan's multiple range test (P<0.05).

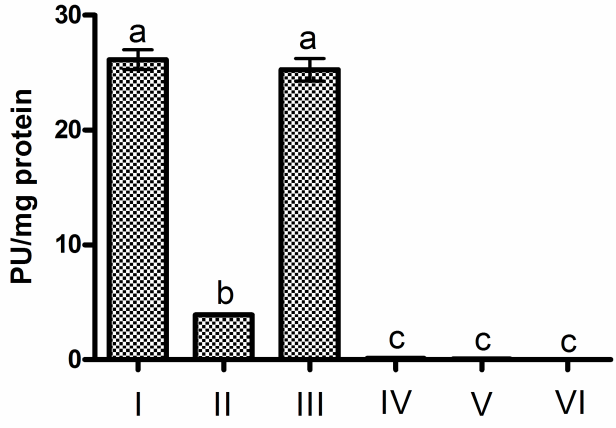

Figure 3.7- The inhibitory activity of red kidney bean seed soluble proteins (4 µg) against extracellular protease of *Alternaria alternata*. **I**= Extracellular proteins of the fungus, **II**= Extracellular proteins of the fungus + soluble proteins of red kidney bean seed, **III**= BSA + fungal proteins, **IV**= Just BSA, **V**= Just bean seed soluble proteins and **VI**= Enzyme control. Values labelled with the same letter do not differ significantly according to Duncan's multiple range test (P<0.05).

3.3.2.2.2- Inhibitory activities of α-amylases of porcine pancreas and *Aspergillus oryzae*

The level of amylase activity of *Aspergillus oryzae* was not significantly different in the presence or absence of crude red kidney bean seed extract while that of porcine pancreatic α-amylase was inhibited by 66.9% with added red kidney bean seed extract (Figs 3.8 and 3.9).

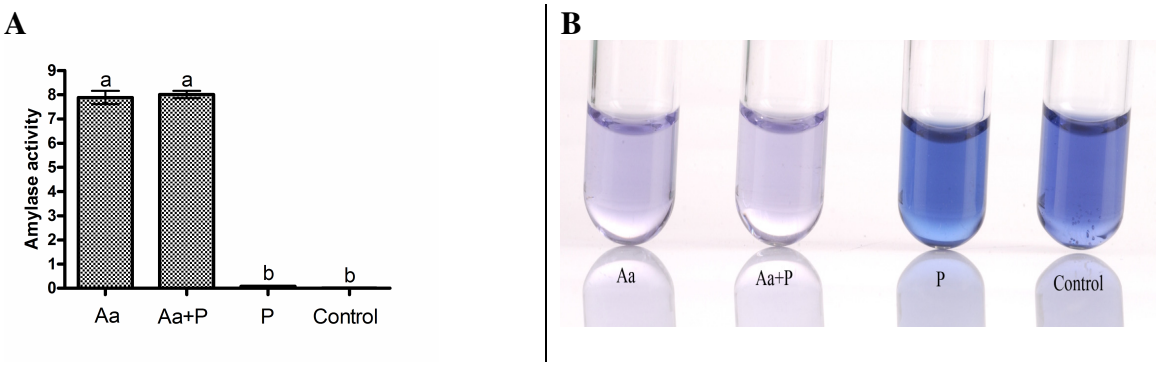

Figure 3.8- Effect of soluble proteins (30 µg) of red kidney bean seed on *Aspergillus oryzae* amylase (**A**). **Aa=** *Aspergillus oryzae* amylase, **P=** Seed soluble proteins. Values labelled with the same letter do not differ significantly according to Duncan's multiple range test ($P<0.05$). **B-** Visual comparison of the inhibitory activity at the end of enzyme reaction in test tubes.

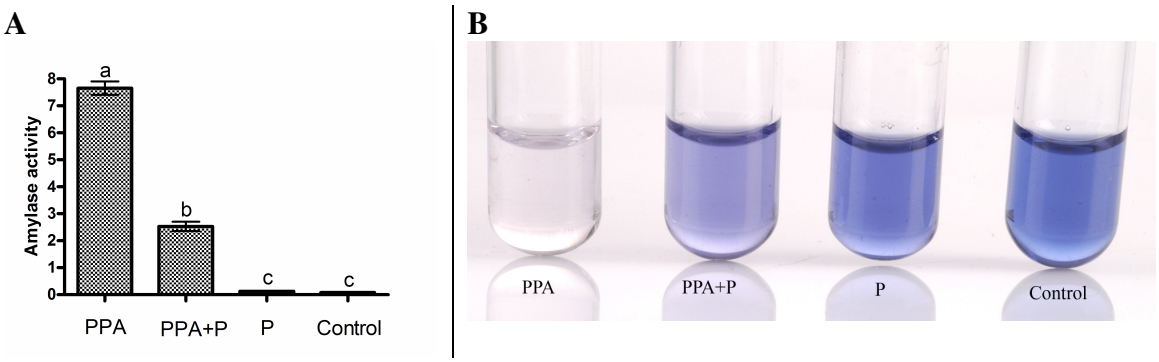

Figure 3.9- Effect of soluble proteins (1 µg) of red kidney bean seed on porcine pancreatic α-amylase (PPA) (**A**). **P=** Seed soluble proteins. Values labelled with the same letter do not differ significantly according to Duncan's multiple range test (P<0.05). **B-** Visual comparison of the inhibitory activity at the end of enzyme reaction in test tubes.

3.3.2.2.3- Bovine trypsin inhibitory activity

Soluble proteins in crude red kidney bean seed extracts (but not another protein BSA which was used as a control) were able to reduce the activity of bovine trypsin by 35.6% (Fig. 3.10).

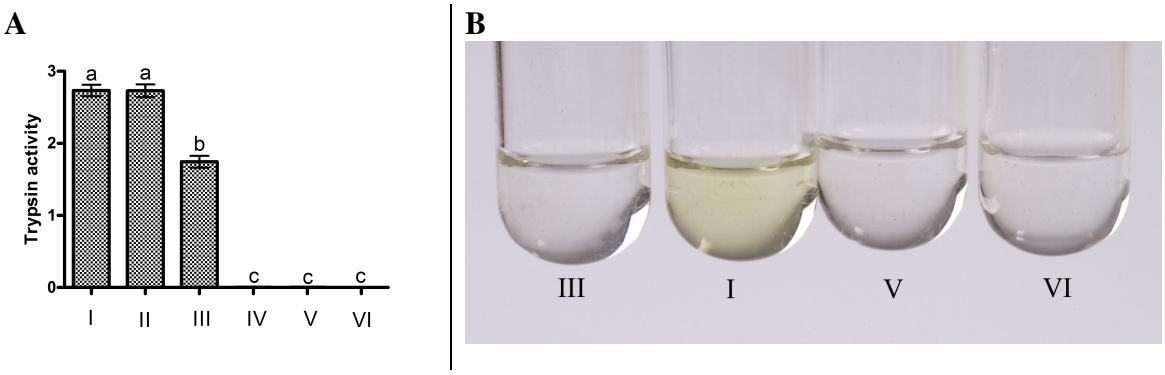

Figure 3.10- Effect of soluble proteins (10 µg) of red kidney bean seed on bovine trypsin (**A**). **I=** Bovine trypsin, **II=** Bovine trypsin + BSA, **III=** Bovine trypsin + bean seed soluble proteins, **IV=** Just BSA, **V=** Just bean seed soluble proteins, **VI=** Control. Values labelled with the same letter do not differ significantly according to Duncan's multiple range test (P<0.05). **B-** Visual comparison of the inhibitory activity at the end of enzyme reaction in test tubes.

3.3.2.3- Antifungal activity *in vitro*

Crude red kidney been seed extracts showed antifungal activity *in vitro* against *Alternaria alternata*, *Cladosporium fulvum* and *Verticillium* sp. among all the fungi tested as described in section 2.1 (Table 3.2). No inhibitory crescent was formed by other fungi in response to the seed extracts.

Table 3.2- *In vitro* antifungal activity of red kidney bean soluble proteins (15 µg) against different fungi.

Fungi	Inhibition crescent (mm)
Alternaria alternata	1.96±0.05
Cladosporium fulvum	1.71±0.028
Verticillium sp.	1.53±0.05
Control	0.000

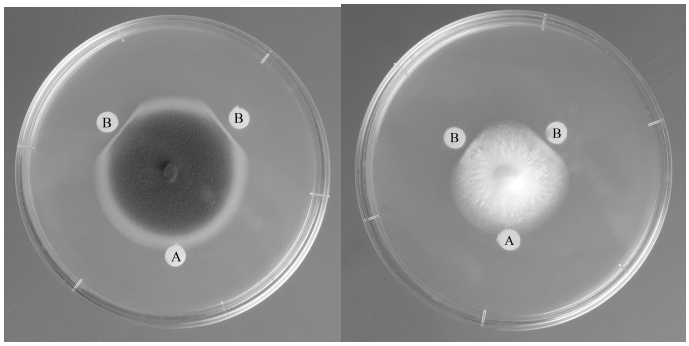

Figure 3.11- Effect of crude bean seed extracts containing 15 µg soluble proteins on growth of *Cladosporium fulvum* (left) and *Verticillium* sp. (right). (**A**)- Control (buffer), (**B**)- Bean seed extracts.

3.3.2.4- Melanin induction in submerged cultivation of *Alternaria alternata*

Crude bean seed extracts were able to induce a significant level of melanin accumulation in *Alternaria alternata* grown in PDB medium compared to

control (Fig. 3.12A). The fungal colony treated with crude bean seed extracts exhibited a darker colour which was clearly detectable even before melanin extraction (Fig. 3.12B).

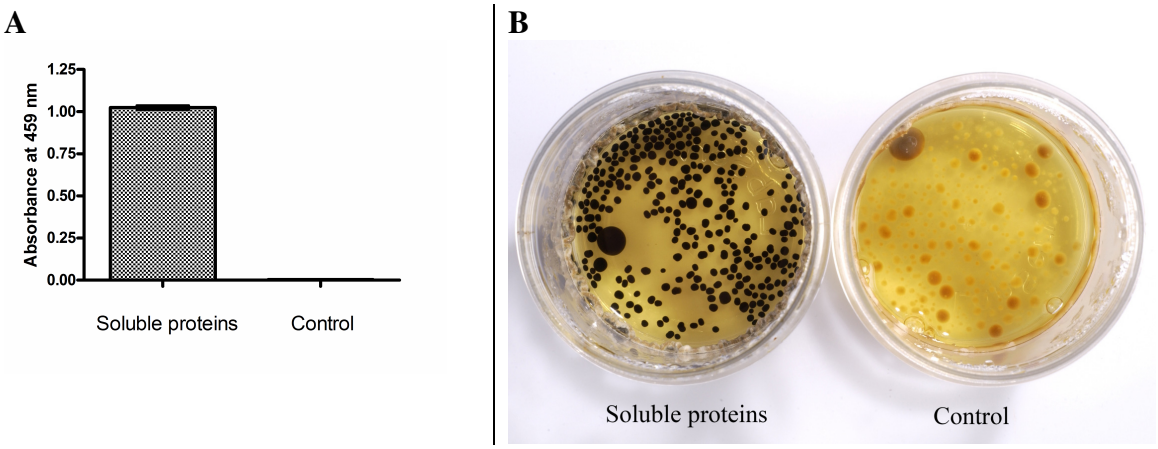

Figure 3.12- Effect of red kidney bean seed extracts containing soluble proteins (100 µg) on melanin accumulation in *Alternaria alternata* (**A**). **B-** Visual inspection of melanin accumulation in *Alternaria alternata*.

3.3.3- Studies of antifungal proteins

3.3.3.1- Purification and assessment

An AFP was purified from both embryonic axis and cotyledons of the red kidney bean. It showed *in vitro* antifungal activity against *Alternaria alternata* (Fig. 3.13). It was not adsorbed on a DEAE-cellulose column but was absorbed on both Affi-gel blue gel and CM-Sepharose columns. From each column two fractions were obtained and the last bound fraction on the CM-Sepharose column was collected. When this fraction with antifungal activity against *A. alternata* was incubated with trypsin for 1 h at 37^0C, its antifungal activity was completely abolished.

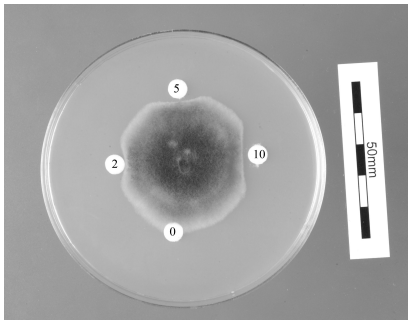

Figure 3.13- Antifungal activity of different concentrations (0, 2, 5 and 10 µg) of antifungal protein isolated from red kidney bean seeds against *Alternaria alternata*.

3.3.3.2- Electrophoresis and identification of the AFP protein isolated from red kidney bean seed

Following SDS-PAGE, the purified AFP of red kidney bean seed was detected as a single band with a molecular mass of 33 KDa (Fig. 3.14).

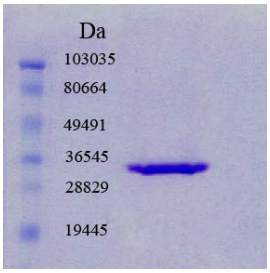

Figure 3.14- SDS-PAGE analysis of 12 µg antifungal protein purified from red kidney bean seeds using different column chromatographic procedures.

The N-terminal sequence of the AFP was found to be identical to the dark red kidney bean hemagglutinin which is devoid of antifungal activity and the flageolet bean antifungal protein which also exhibits hemagglutinating activity. The sequence of the purified AFP was also found to be similar to that of red kidney bean hemagglutinin (Table 3.3).

Table 3.3- Alignment of the N-terminal sequence of red kidney bean AFP isolated in the present study with other hemagglutinins and AFPs purified from other *Phaseolus vulgaris* cultivars. FBH- Flageolet bean hemagglutinin (source: *Phaseolus vulgaris* cv. Flageolet bean), DRKBH- Dark red kidney bean hemagglutinin (source: *Phaseolus vulgaris* cv. Dark red kidney bean), RKBH- Red kidney bean hemagglutinin (*Phaseolus vulgaris* cv. Red kidney bean) and FBN35H- French bean number 35 hemagglutinin (*Phaseolus vulgaris* cv. French bean number 35).

Name	N-terminal sequence	AF activity	Reference
AFP (in this study)	S N D I Y F N F	+	N/A
FBH	S N D I Y F N F Q R	+	Xia and Ng, 2005
DRKBH	S N D I Y F N F Q R	-	Xia and Ng, 2006
RKBH	A N Q T S F N F Q R F D	+	Ye *et al*., 2001b
FBN35H	A T E T Y S A F Q R	+	Lam and Ng, 2010

3.3.3.3- Fungistatic effect of the purified AFP

In YPG medium containing the AFP (40 μg/ml) purified from red kidney bean seeds, germination of conidia of *A. alternata* was inhibited. However, when the medium containing AFP was replaced with fresh medium without AFP, conidial germination comparable to control was observed. This suggested that the AFP is a fungistat not a fungicide as the toxicity of AFP was reversible.

3.3.3.4- Morphological changes in the fungus in response to addition of red kidney bean AFP

Microscopic observations revealed morphological changes in hyphae of *A. alternata* (Fig. 3.15). The fungal hyphae treated with AFP were poorly developed, short and swollen whereas those in the control were intact, well developed and normal.

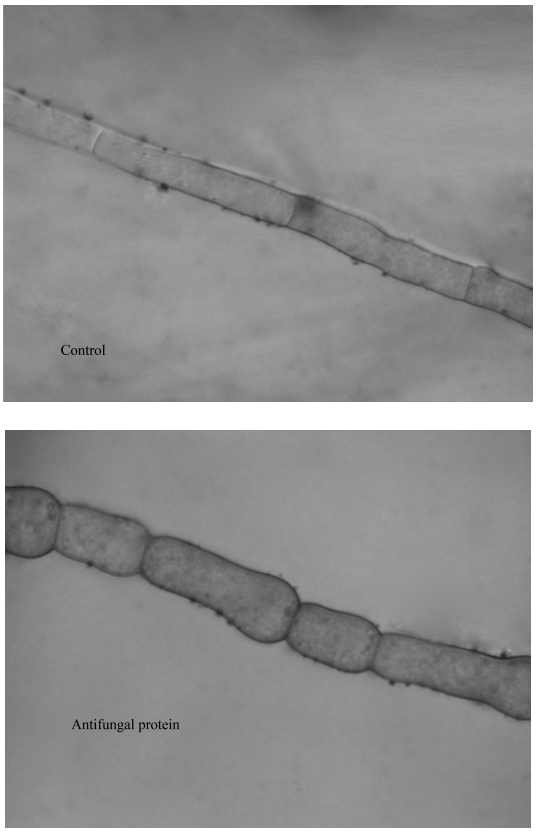

Figure 3.15- Morphological changes induced in fungus by red kidney bean antifungal protein (×1000).

3.3.3.5- Spore germination assay

Percent spore germination of *A. alternata* was decreased significantly in the presence of purified red kidney bean AFP in a concentration-dependent manner (Fig. 3.16). BSA was used as a control and did not have any effect on conidia germination.

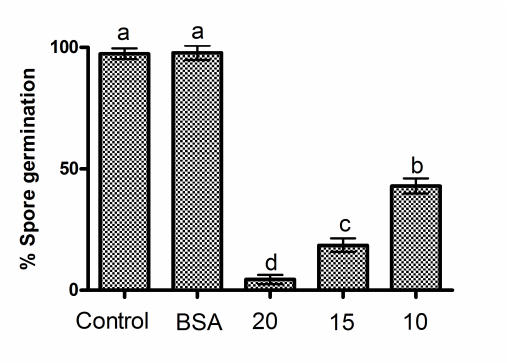

Figure 3.16- Effect of different concentrations of purified red kidney bean AFP (10, 15 and 20 µg) and bovine serum albumin (50 µg) on germination of *Alternaria alternata* conidia. Each assay was carried out in triplicate. Values labelled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

Figure 3.17- A germinating spore of *Alternaria alternata* (×1000)

3.3.3.6- Probing for additional bioactive properties of antifungal protein from red kidney bean seeds

The AFP purified from red kidney bean seeds in this study did not show either chitinolytic or β-1, 3- glucanase activities. It was also not able to inhibit the activity of bovine trypsin, PPA or protease from *Alternaria alternata*. However, unexpectedly it was able to enhance PPA activity *in vitro* as significantly more reducing sugars were released from starch

hydrolysis when PPA was incubated together with the AFP compared to PPA incubated alone (Fig. 3.17).

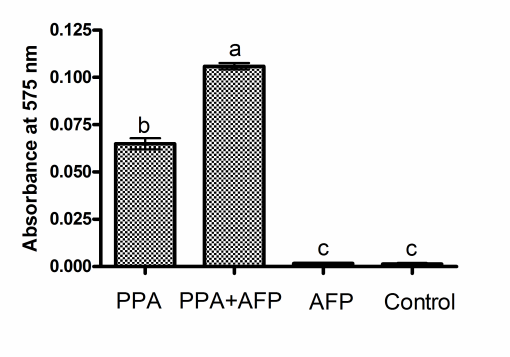

Figure 3.18- Stimulatory effect of purified red kidney bean AFP (4 μg) on porcine pancreatic α-amylase (PPA). **AFP** = Red kidney bean purified antifungal protein. Higher absorbance values in the graph show more starch hydrolysis or more sugar release. Values labelled with the same letter do not differ significantly according to Duncan's multiple range test ($P<0.05$). Control without addition of AFP or PPA.

3.4- Discussion

Among all fungi tested in the preliminary experiments, *Alternaria alternata* was selected for further bioactivity studies in this project as its mycelial growth was found to be inhibited by crude protein extracts of red kidney bean seed. Many phytopathogenic microorganisms have been reported to secrete a variety of extracellular enzymes which are important in pathogenesis (Habib and Fazili, 2007). In the present study, during growth *in vitro*, *A. alternata* was able to secrete extracellular amylase and protease. This is consistent with previous studies (Patil and Shastri, 1985; Abdel-Sater and Ismail, 1993; Chung and Hwang, 1996; Dunaevskii *et al*., 1996; Shafique *et al*., 2010). Fructose added to Czapek Dox medium was previously shown to enhance production of protease by *A. alternata* (Patil

and Shastri, 1985). One neutral and two alkaline proteases were isolated and purified from extracellular extracts of *A. alternata* (Patil and Shastri, 1985).

In the present study, soluble proteins of red kidney bean seeds were found to inhibit activity of protease secreted from *A. alternata* as well as that of a commercially prepared bovine trypsin. Although trypsin inhibition by heat-stable extracts of red kidney bean seeds has been reported before (Wati *et al*., 2009; Alves *et al*., 2010), there was no prior report on inhibition of extracellular protease activity of *A. alternata* by red kidney bean seed extracts. The protease produced by *A. alternata* was reported in a previous study as a trypsin-like enzyme which belongs to the group of serine proteinases of which bovine trypsin is a member (Rawlings and Barrett, 1994; Dunaevskii *et al*., 2006). It can be assumed that trypsin inhibitors in red kidney bean seeds can also inhibit the extracellular protease activity of *A. alternata*. This would be similar to a previous study showing that trypsin and chymotrypsin inhibitors isolated from soy and bean seeds were able to inhibit extracellular protease activity of *Fusarium solani* (Valueva and Mosolov, 2004). In another research, the inhibitors of trypsin and chymotrypsin isolated from kidney bean seeds (*Phaseolus vulgaris* cv. Tweed Wonder) did not inhibit activities of proteolytic enzymes produced by *Colletotrichum lindemuthianum* grown in alkaline media but crude extracts of the seed did. The purified protease inhibitor (against *C. lindemuthianum*) from bean seeds was inactive against another fungal protease from *Aspergillus oryzae*. Interestingly, inhibitors of both bovine trypsin and fungal (*C. lindemuthianum*) protease had similar isoelectric points and their amino acid composition resembled each other (Mosolov *et al*., 1979). More extensive examples can be found in Mosolov *et al*., 2001a.

Thus it seems to be a good idea to work in the future on purification of the protease from *A. alternata* and its inhibitor from red kidney bean seeds to clarify whether the protease inhibitor from red kidney bean is a trypsin inhibitor or not.

Fungal proteases may degrade receptor proteins in plants and play a role in plant-microbe interactions (Mosolov *et al*., 1979) as they may also cleave antimicrobial proteins of plants (Mosolov *et al*., 2001a). Therefore, inhibition of fungal proteases can increase plant resistance to fungal attack. As protease inhibitors have been reported to be involved in plant resistance against pathogens (Valueva and Mosolov, 2004; Dunaevskii *et al*., 2005a; Hermosa *et al*., 2006) or may take part in plant protection (Mosolov *et al*., 1979) it would seem possible that our purified AFP might be capable of inhibiting protease activity of *A. alternata* and consequently contribute to its antifungal activity. In a medium like PDA containing both sugar and starch, a fungus might not rely solely on its extracellular protease enzyme to survive as it can consume carbohydrates. This is consistent with a former report showing that *A. alternata* grown in a medium without casein (like PDA used in the antifungal tests of the present study) does not produce protease (Dunaevskii *et al*., 2006). Therefore, our seed protease inhibitor cannot be considered as an agent responsible for suppression of *A. alternata in vitro* as no extracelluar protease was present when the fungus was grown on PDA (data not shown). Consequently antifungal activity *in vitro* was shown without an accompanying protease inhibitory activity and the suggestion that inhibition of the fungal growth on PDA plates by crude extracts of red kidney bean seed was mediated through inhibiting its extracellular protease is not reasonable. However, the fungal protease inhibitor in crude red kidney

bean seed extracts might have a second function involved in antifungal activity which is distinct from its ability to inhibit the fungal protease. More experiments are needed after AFP purification to determine whether the antifungal activity in red kidney bean seed is associated with its inhibitory activity against the fungal protease or not.

From studies on *in vitro* antifungal activity a question arises as to whether the protease inhibitor present in crude red kidney bean extracts could be involved in plant resistance *in vivo* by inhibiting extracellular fungal protease. In corn, for example, a trypsin inhibitor has been found to inhibit *Aspergillus flavus* α-amylase as well. This bifunctional protein was able to inhibit fungal hyphal growth as well as conidia germination. The fungal growth inhibition was related to the α-amylase inhibitory activity of the protein even though it was suggested this might not be enough for complete inhibition of fungal growth. When exogenous α-amylase or 5% glucose was added to the medium, the protein lost its antifungal activity. It was also found that 10% PDB (potato dextrose agar) medium was able to provide sufficient simple sugars for fungal growth suggesting that no additional external α-amylase was needed for fungal growth (Chen *et al*., 1999).

In the present study, it was necessary to characterize the purified AFP as it could be a multifunctional protein with different bioactivities and mode of actions on *A. alternata*. Because in crude protein extracts of red kidney bean there were antifungal activity as well as protease inhibitory activity of *A. alternata*, it could be suggested that the protease inhibitory activity of the purified AFP from red kidney bean might have a secondary activity involved in suppression of *A. alternata* growth *in vitro*. However, the result of the

present study showed that the purified AFP from red kidney bean seed had no protease inhibitory activity, suggesting that the AF activity of crude red kidney bean extracts was independent of the inhibitory activity of *A. alternata* protease. It would be interesting to study in future whether the protease of *A. alternata* is able to degrade the purified AFP or not.

Soluble proteins of red kidney bean seeds did not inhibit fungal amylases including those of *A. alternata* and *Aspergillus oryzae* but inhibited strongly the activity of pancreatic α-amylase (PPA). Previously it was also found that there was no inhibitory activity in seed proteins of the common bean against amylases of *Fusarium* spp., *Sclerotinia* spp. and *Aspergillus oryzae* (Powers and Whitaker, 1978; Kluh *et al*., 2005). In contrast, presence of inhibitors against α-amylases from *F. verticillioides*, *Aspergillus flavus* and PPA in corn seeds has been reported before (Figueira *et al*., 2003a and b). Interestingly the inhibitor reduced conidia germination in *F. verticillioides* (Figueira *et al*., 2003b). It seems that more research is needed regarding amylases and structures of their inhibitors from fungi and seeds.

It had been reported before that soluble proteins of red kidney bean seed were able to inhibit PPA (Grossi de Sa *et al*., 1997; Santimone *et al*., 2004). Preincubation of PPA and inhibitor (red kidney bean αAI-1) is necessary before adding the substrate to observe inhibitory effect (Koukiekolo *et al*., 1999; Santimone *et al*., 2004). It was found that inhibitor and PPA must be preincubated for at least 8 min in the present study (data not shown). Formation of a complex between inhibitors of red kidney bean (including αAI-2 and αAI-1) and α-amylases of Mexican bean weevil and PPA,

respectively, was time-dependent (Powers and Whitaker, 1978; Grossi de Sa *et al*., 1997). Inhibitors and enzymes from different sources have been preincubated for different times. For example αAI-2 and Mexican bean weevil α-amylase were preincubated for 1 h in a previous study to observe any significant inhibition (Grossi de Sa *et al*., 1997). Preincubation of *F. verticillioides* amylase and an inhibitor from corn seed was also necessary as it was found that enzyme-inhibitor complex formation was maximized after 30 min of preincubation (Figueira *et al*., 2003b). In the present study, preincubation of crude red kidney bean seed extracts and *A. alternata* amylase up to 20 min did not reveal any activity against the fungal enzyme. This was also observed in another study on inhibition of amylases of *Fusarium* spp. and *Sclerotinia* spp. (Kluh *et al*., 2005). Thus it is important to check whether longer preincubation times might be needed to detect any inhibitory activity from red kidney bean seed against fungal amylases.

Chitinase activity was detected in crude red kidney bean seed extracts as reported before (Ramos *et al*., 1998). However, it is not known if bean seed chitinase also has antifungal activity. A thermostable chitinase isolated from Canadian cranberry beans (*Phaseolus vulgaris*) was shown to inhibit mycelial growth *in vitro* of *Pythium aphanidermatum*, *Physalospora piricola*, *Botrytis cinerea* and *Fusarium oxysporum* (Wang *et al*., 2009b). Based on the published literature, there is no chitin in the cell wall of *Pythium aphanidermatum* and the fungal cell wall is composed of cellulose instead of chitin (Blaschek *et al*., 1992; Alexopoulos *et al*., 1996). Therefore, the antifungal activity of the isolated chitinase of cranberry bean might be different from its chitinolytic activity.

Crude red kidney bean seed extracts were also found to have β-1, 3-glucanase activity. There was no prior report about β-1, 3- glucanase activity in red kidney bean seeds while other studies showed a β-1, 3- glucanase with antifungal activity in cowpea seeds as well as its presence in bean leaves in response to biotic and abiotic treatments (Hughes and Dickerson, 1991; Mauch *et al*., 1992; Gomes *et al*., 1996). Future work can investigate if the purified glucanase from red kidney bean seeds has antifungal activity.

Crude red kidney bean seed extracts induced melanization in mycelia of *A. alternata*. Melanogenesis in fungi can be induced in response to stress factors including toxic metals, hyperosmotic conditions, pH shock, UV irradiation, desiccation, antagonistic microbes, nutrient deprivation (Henson *et al*., 1999; Thomma, 2003) and heat shock (Thompson *et al*., 2008). Some of these factors such as nutrient deprivation, osmotic and moisture stresses, desiccation and near ultraviolet light have been reported as conidiation inducers in fungi as well (Sokolovsky *et al*., 1992; Li *et al*., 1997; Pascual *et al*., 1997; Masangkay *et al*., 2000; Chovanec *et al*., 2001; Roncal and Ugalde, 2003; Fischer and Kues, 2006; Krasniewski *et al*., 2006; Carvalho *et al*., 2008; Nemcovic *et al*., 2008; Xu *et al*., 2009). A relationship between melanization and sporulation has been reported before (Uehara *et al*., 1995; Calvo *et al*., 2002). Here, we hypothesized that soluble proteins of red kidney bean seeds can induce conidiation in *A. alternata* (see chapter 5). Hydrogen peroxide was able to decolour melanin extracted from *A. alternata* (data not shown) and this is consistent with previous investigations (Butler and Day, 1998). Future work can focus on gene expression involved in

melanin production in fungi during conidiation and relationship of this to mechanism of conidiation. Also as some of compounds such as glyphosate (Nosanchuk *et al*., 2001), tricyclazole, PCBA, pyroqilon, coumarin, phthalide, tetrachlorophthalide (Woloshuk and Sisler, 1982; Wheeler and Klich, 1995; Hamilton and Gomez, 2002) have been reported as melanin biosynthesis inhibitors, it would be worthwhile to work on effects of these compounds on melanization in *A*. *alternata* in the presence of red kidney bean seed extracts.

The present study was the first report showing that the AFP in crude and purified extracts of red kidney bean seeds had activity against growth of *Alternaria alternata in vitro*. The AFP of red kidney bean seeds did not show activity against *Fusarium oxysporum* mycelial growth as reported previously (Xia and Ng, 2005). The AFP was a heat stable protein as it remained soluble after a protein isolation step involving heating at 70^0C for 10 min. It was also degraded by trypsin as following 1 h incubation at 37^0C with bovine trypsin the AFP lost its antifungal activity. These results are consistent with that of Xia and Ng (2005). The method used here for AFP purification was also similar to that used to purify the flageolet bean antifungal protein. N-terminal sequence (8 residues) of AFP of red kidney bean was identical to that of dark red kidney bean hemagglutinin which had no known activity against fungi (Xia and Ng, 2006) as well as flageolet bean antifungal protein which was known to have hemagglutinating activity (Xia and Ng, 2005). It seems that the AFP isolated in the present study was more similar to that isolated from flageolet bean seeds than the dark kidney bean seed hemagglutinin. N-terminal sequence of the purified AFP in the present

study also showed 60 and 40% similarity, respectively, to red kidney bean hemagglutinin (Ye *et al*., 2001b) and French bean number 35 hemagglutinin (Lam and Ng, 2010). Thus it would be worthwhile to determine if the purified AFP in the present study also has hemagglutinin activity in future studies. Interestingly, all the aforementioned proteins except red kidney bean hemagglutinin were not able to inhibit mycelial growth of *Fusarium oxysporum in vitro*. The red kidney bean hemagglutinin has been reported to inhibit this fungus at a concentration equal to 300 μg/AFP. The decision as to which gene of AFP could be used to generate transgenic plants with improved fungal resistance should not rely solely on studies of *in vitro* effect of an AFP. More work on purified AFPs including their whole sequences are required for further studies. The cDNAs of AFPs can then be cloned in *Escherichia coli* for production of large quantities of AFPs. This would enable more studies about their activities against different organisms or even can be assessed as an antifungal compound to be sprayed on plants.

Fungistatic or fungicidal effect of the red kidney bean AFP had not been investigated before. It was shown that an AFP was fungistatic at lower concentrations (below the minimal inhibitory concentration) but acted as a fungicide at higher concentrations (Theis *et al*., 2005). Here, the conidia of *A. alternata* were not killed after treatment with the red kidney bean AFP. They were able to germinate after removal of the AFP suggesting that the AFP was a fungistat. Similarly, an AFP with trypsin/chymotrypsin inhibitory activity purified from buckwheat seeds was found to suppress spore germination and mycelial growth of *A. alternata* (Dunaevskii *et al*., 1997).

Morphological changes and deformation were observed in *A. alternata* mycelia treated with the red kidney bean AFP showing poorly developed mycelium, swollen and misformed hyphae. These changes were also observed in other studies (Singh *et al*., 2007; Kirubakaran *et al*., 2008; Meyer, 2008). Mechanisms of action of antifungal proteins are multiple and complex. For example they might have an intracellular target while they could permeabilize membrans (Leiter *et al*., 2005; Van der Weerden *et al*., 2008). More research with different techniques including transmission electron microscopy (for example, using immunogold-labeled AFP) (Kim and Chung, 2004; Leiter *et al*., 2005; Theis *et al*., 2005) and confocal laser scanning microscopy (for example, using labled-protein) (Van der Kraan *et al*., 2005) can help to find out more about internalization and localization of AFP into fungal cells and other ultrastructural changes in the fungus including increased vacuolization or plasma membrane permeabilization. Our knowledge about the mode of actions of AFP from seeds is still very limited. This can be a reason why AFPs have not been used widely in food preservation or in control of pathogenic fungi (Theis *et al*., 2005)

Chapter 4- Studies on α-Amylase Stimulators in Red Kidney Bean Seeds

4.1- Introduction

Dry mature red kidney bean seeds (*Phaseolus vulgaris*) contain biologically active proteins including α-amylase inhibitors (α-AI) against insect and mammalian α-amylases but not plant α-amylases (Santimone *et al*., 2004). Porcine pancreatic α-amylase (PPA), a commercially available mammalian α-amylase, is widely used to facilitate determination and purification of α-amylase inhibitors in extracts of seeds (Moreno *et al*., 1990; Payan 2004; Santimone *et al*., 2004; Mosca *et al*., 2008). Another group of abundant biologically active seed proteins in *P. vulgaris* consists of lectins, particularly phytohemagglutinin (PHA). The five tetrameric isoforms of PHA are mainly comprised of combinations of two types of subunits, the PHA-E (erythroagglutinating phytohemagglutinin) and PHA-L (leucoagglutinating phytohemagglutinin) (Dao-Thi *et al*., 1996; Hamelryck *et al*., 1996; Biswas and Kayastha 2004; Morari *et al*., 2008). Both subunits have different biological activities and arose through duplication of an ancestral gene (Leavitt *et al*., 1977; Hoffman and Donaldson, 1985).

The α-AI (a truncated lectin) (Rouge *et al*., 1993) and PHA from *P. vulgaris* have been shown to be anti-insect proteins (Franco *et al*., 2002; Kluh *et al*., 2005) and mitogens of lymphocytes (Shi *et al*., 2007), respectively. However, their *in vivo* functions apart from being a part of the nitrogen store

for seed germination remain unclear (Morari *et al*., 2008). One previous report showed that PHA-P purified from *P. vulgaris* was able to enhance activity of PPA *in vitro* (You and Chang 1992). This was not observed in the previous studies using impure PHA containing α-AI as a contaminant (Thompson and Gabon, 1987; Fish and Thompson, 1991). Instead, these studies had erroneously implicated that PHA could contribute to a lower rate of digestion of starch from legumes than that from cereals (Thompson, 1988). However, the implications for α-AI and PHA-L (or other isoforms of PHA) present together in the same seed extracts and their possible *in vitro* interactions on the detection and quantification of α-AI have not been the specific focus of any previous studies. In other words, it has not been investigated before whether these biologically active proteins are present in the same seed extracts might influence their determination in *in vitro* assays.

In this chapter we focused on red kidney bean α-amylase stimulators (these proteins were called "stimulators" here because of their promotive effect on porcine α-amylase) and their distribution in seed parts. In the literature, there was a paper (You and Chang, 1992) on proteins isolated from red kidney bean seeds that were able to promote porcine amylolytic activity but the work was not continued later and no more information was found except a few papers about stimulatory activity on amylolysis in insects or increasing amylase secretion in different animals (Macedo *et al*., 2007; Grant *et al*., 1997; Kordas *et al*., 2000; Baintner *et al*., 2004b). There was a huge gap in this area of knowledge. There is also a paucity of information about the effect of extracts from the embryonic axis of red kidney bean on PPA or other non-plant amylases.

It was hypothesized that since proteinaceous α-amylase inhibitor (α-AI) and stimulator might be extracted simultaneously from red kidney bean seeds (*Phaseolus vulgaris*) their *in vitro* interactions might influence their detection and quantification. In preliminary experiments, a fraction obtained from partial purification of red kidney bean seed extracts using DEAE-cellulose and Affi-gel blue gel columns was found to enhance PPA activity. It was then found to contain a protein of apparent M_r of approximately 31-32 KDa and was identified to be PHA-L after MALDI mass spectrometry. It was of interest to determine if PHA-L might contribute to enhancement of PPA activity by the partially purified bean protein fraction.

When PHA was present together with α-AI in an extract, detection and interpretation of assay results of inhibitor (α-AI) and stimulator (PHA) of PPA activity was complex, a situation likely unrealized in previous works. In detection of α-amylase isoinhibitors in some seed parts or plant parts, it was possible that their inhibitory activity was masked by the stimulators and consequently their inhibitory activity was not detectable by either of the test tube methods (sugar assay or starch-iodine complex method). Therefore, besides these methods the native gel method (refer to 2.5.2.1) was also applied to see whether amylase isoinhibitors were present in the extracts of different seed parts or not.

The importance of these findings is discussed in relation to further studies on α-AI and stimulator of α-amylases *in vitro*. The occurrence of proteinacous α-amylase stimulator in seeds and possibly other plant parts could be widespread. Establishing this is an important prerequisite for designing

future investigations to gain new insights into the little known physiological significance of α- amylase stimulator at least in red kidney bean seeds.

4.2- Materials and methods

4.2.1- Extraction and preparation of extracts for assay of α-amylase inhibitory activity

Red kidney bean seeds (7 g) were soaked in water and extracted as described in 2.2. The extracts of red kidney bean cotyledons prepared as described in 2.2 were heated in a boiling water bath for 10 min. These extracts as well as those from the cotyledons, embryonic axis and seed coat were used for assay of α-amylase inhibitory activity (2.7.3.1 and 2.7.3.2). In-gel detection (see section 2.5.2.1) was also carried out to detect different α-amylase isoinhibitors in the extracts.

4.2.2- Isolation, SDS-PAGE and identification of PHA-L as an amylase stimulator

Ammonium sulphate was added to an extract of red kidney bean cotyledons and was fractionated to 85% saturation. The precipitate was dialysed overnight against 20 mM phosphate buffer at pH 6.8 and applied to an anionic exchange DEAE cellulose (Cat. D-6418, Sigma, St. Louis, Missouri, USA) column equilibrated with the same buffer. The fraction of unbound proteins from this column was dialysed against 20mM tris-HCl buffer at pH 7.3 before it was applied to an affinity column with Affi-Gel Blue gel (Bio-Rad) equilibrated with the tris-HCl buffer. Unadsorbed proteins were eluted from this column with the same buffer before the bound proteins were eluted

with 0.2 M NaCl in the same buffer. The adsorbed fraction (denoted as 'Affi-fraction' in this project) was assessed for its effect on PPA as described in the in-solution determination of α-AI activity and analysed using SDS-PAGE as described before (2.5.1).

A protein band of apparent M_r of 31-33 KDa was chosen for MALDI mass spectrometric analysis. It was excised from SDS-PAGE and then sent to the Centre for Protein Research, Department of Biochemistry, Otago University (Dunedin, New Zealand) to be analysed on a 4800 MALDI tandem Time-of-Flight Analyser (MALDI TOF/TOF, Applied Biosystems, MA) after in-gel digestion with trypsin according to the method described by Shevchenko *et al.*1996 (for methods see the Appendices). To help identify the protein, MS/MS data generated were searched against the UniProt/SWISS-PROT amino acid sequence database using the Mascot search engine. (http://www.matrixscience.com). A commercial PHA-L (Sigma L2769) was obtained for the present study to check its effect on PPA activity.

4.2.3- Purification of PHA

Extracts of red kidney bean cotyledons (20 g) prepared as described in 2.2 were fractionated with ammonium sulphate. The precipitate obtained between 0-85% saturation was redissolved in 2 ml of potassium phosphate buffer (10 mM, pH 6.9) and dialyzed against the same buffer. The dialyzed extract (50 ml) was mixed with 5 ml porcine thyroglobulin-agarose (Cat. T4398, Sigma, St. Louis, USA) and tumbled at 4^0C overnight. This mixture was washed in a column (1 cm × 12 cm) with the phosphate buffer containing 1 M NaCl following the method of Ren *et al.* (2008a) and PHA was eluted from the column with 0.05 M glycine (Cat. 161-0724, Bio-Rad,

Hercules, California, USA)-HCl pH 3.0 containing 0.5 M NaCl. The eluted protein fraction was desalted by dialysing against 100 mM sodium acetate buffer, pH 5.6 and freeze dried.

4.3- Results

4.3.1- In-gel detection of α-amylase isoinhibitors

PPA inhibitory activity extracted at pH 3.8 from the cotyledons and embryonic axis of mature red kidney seeds remained soluble after heating at 70^0C for 10 min and active in non-denaturing polyacrylamide gels for detection of isoinhibitors of PPA (Fig. 4.1). There were at least 4 prominent bands of PPA inhibitory activity in the extracts of both the cotyledons and embryonic axis. There was no detectable α-AI in seed coat extracts.

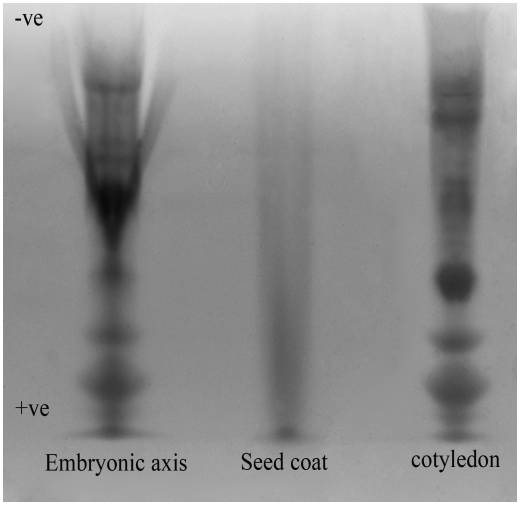

Figure 4.1- Detection of isoinhibitors of PPA activity following non-denaturing PAGE of extracts from the cotyledon, seed coat and embryonic axis of red kidney bean seeds. Equal amount of protein (30 μg) was loaded in each lane.

When cotyledonary extracts were boiled for 10 min most bands of PPA isoinhibitor were no longer detectable (Fig. 4.2).

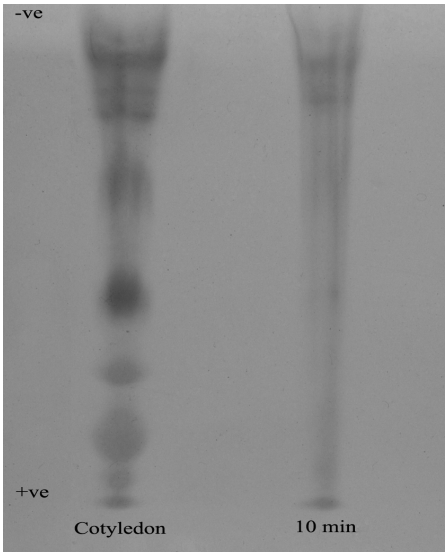

Figure 4.2- Effect of boiling (10 min) of extracts from red kidney bean cotyledons on the profile of isoinhibitors of α-AI following non-denaturing PAGE. Equal amount of protein (30 μg) was loaded in each lane.

4.3.2- In-solution (test tube) experiments

Interestingly, PPA activity in the presence of extracts of the embryonic axis was more than PPA alone (Table 4.1 and Fig. 4.4A and B). Amylase stimulatory activity was not observed when amylase of *Aspergillus oryzae* was used as a source of amylase. Extracts of the cotyledons exhibited inhibitory or stimulatory effect on α-amylase activity depending on whether prior to the α-amylase assay they had been boiled or not. The enhancing effect of the extracts on PPA in the in-solution PPA inhibitor assay was revealed only after the extracts had been placed in a boiling water bath

(100 ± 2^0C) for 10 min. This resulted in the disappearance of most bands of isoinhibitor of PPA (Table 4.1).

No inhibitory activity was detected in the seed coat with either in-gel (Fig. 4.1) or in-solution methods but a high stimulatory activity in seed coat extract was observed (Fig. 4.5). An affinity column (porcine thyroglobulin agarose) commonly used to purify lectins (including a mixture of isoforms of PHA from *Phaseolus vulgaris* seeds) was successful to purify a single protein of approximately 32 KDa on SDS-PAGE (Fig. 4.3) and was devoid of amylase or inhibitory activity against PPA but instead it enhanced PPA activity by close to 70% (Table 4.1).

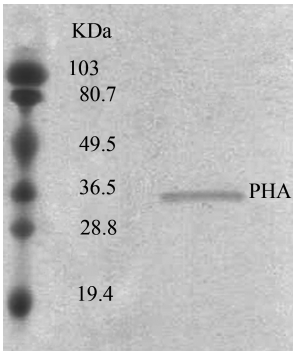

Figure 4.3- Purification of PHA from extracts of red kidney bean cotyledons. An aliquot (5µl) of Bio-Rad prestained molecular weight standards and 8 µg of protein from porcine thyroglobulin-agarose affinity column chromatography were loaded, respectively, for SDS-PAGE.

Table 4.1- Effect of red kidney bean cotyledon extract (1 μg of protein) on porcine α-amylase activity before and after boiling, and in a separate experiment studying effect of PHA (7 μg) purified from red kidney bean cotyledons on α-amylase activity. Control reaction was without α-amylase in the first experiment or PHA in the second experiment. The control reactions resulted in the high absorbance readings (A_{620}) of 1.162 and 1.316 in the respective experiments. Each assay was carried out in triplicate. Mean absorbance values assigned with the same letter do not differ significantly according to Duncan's Multiple Range test ($P<0.05$).

	Treatments	Amylase activity
Cotyledon extract		
	Amylase (PPA)	5.17 ± 0.15b
	Amylase + boiled extract	9.21 ± 0.056a
	Amylase + extract	1.43 ± 0.026c
	Boiled extract (no PPA)	0.01 ± 0.001d
	Extract (no PPA)	0.04 ± 0.0019d
	Control	0.03 ± 0.0017d
PHA		
	Amylase (PPA)	6.55 ± 0.31b
	Amylase + PHA	11.03 ± 0.15a
	PHA	0.013 ± 0.001c
	Control	0.01 ± 0.001c

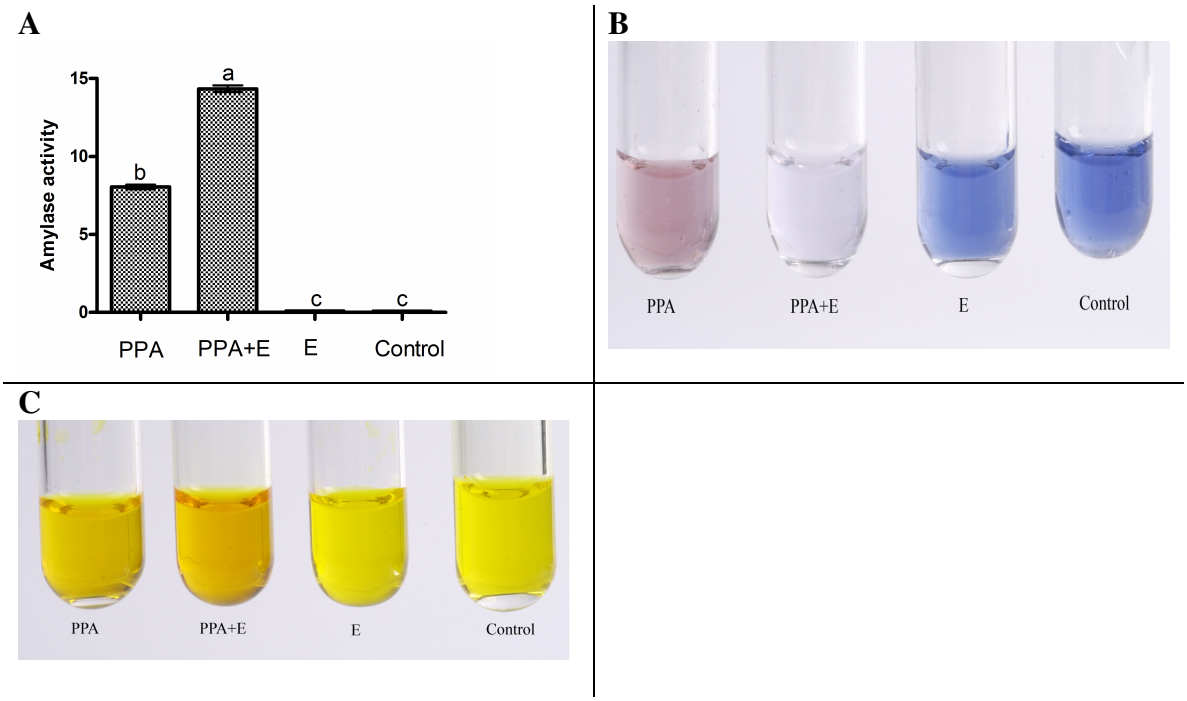

Figure 4.4- Enhancement of porcine pancreatic α-amylase activity (PPA) by embryonic axis extract (0.1 µg protein) (**A**). **E**= Embryonic axis extract. Control reaction was without PPA and embryonic axis extract. Values labelled with the same letter do not differ significantly according to Duncan's multiple range test ($P<0.05$).
B and **C-** Visual comparison after the end of enzyme reaction showing enhancement effect of the extract of red kidney bean embryonic axis on porcine pancreatic α-amylase by two different assays, sugar assay (**C**) and starch-iodine complex method (**B**).

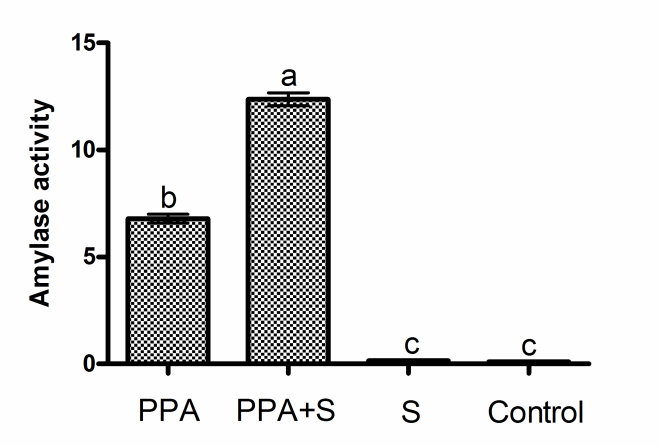

Figure 4.5- Enhancement of porcine pancreatic α-amylase activity (PPA) by red kidney bean seed coat extract (0.1 µg protein). **S**= Seed coat extract. Control reaction was without PPA and seed coat extract. Values labelled with the same letter do not differ significantly according to Duncan's multiple range test ($P<0.05$).

4.3.3- SDS PAGE and identification of PHA-L as a PPA stimulator

In-solution assays of PPA activity in the presence and absence of the fraction partially purified using DEAE-cellulose and Affi-gel blue gel columns (denoted as 'Affi-fraction') showed a significant enhancement effect of Affi-fraction on PPA activity (Fig. 4.6).

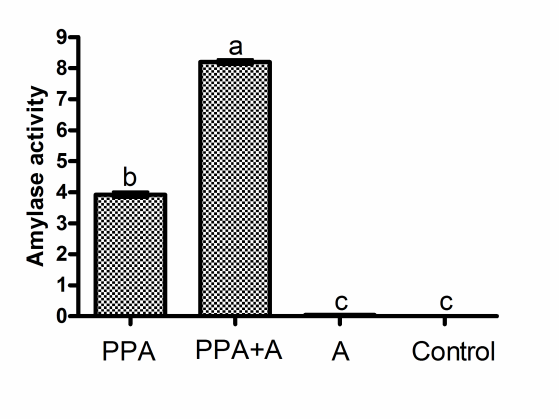

Figure 4.6- Enhancement of porcine pancreatic α-amylase activity (PPA) by Affi-fraction (5 µg protein). **A**= Affi-fraction (partially purified extracts from red kidney bean cotyledons using DEAE-cellulose and Affi-gel blue gel columns. Values labelled with the same letter do not differ significantly according to Duncan's multiple range test ($P<0.05$).

Affi-fraction was resolved into three protein bands with apparent molecular masses of 31, 21 and 14 KDa after SDS PAGE and staining with Coomassie Brilliant Blue (Fig. 4.7). The bands were analysed using MALDI TOF/TOF and the 31 KDa band was identified as PHA-L (score 821) and the 14 and 21 KDa ones were identified as mannose-binding lectins which were also known as the FRIL of *Phaseolus vulgaris* (scores 579 and 713 respectively and the mass spectra data shown in appendices) or PvFRIL (*Phaseolus vulgaris* fetal liver tyrosine kinase 3-receptor interacting lectin).

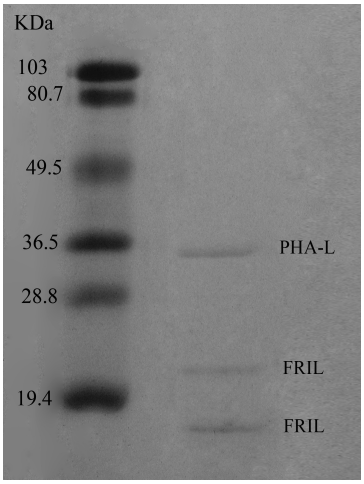

Figure 4.7- SDS-PAGE analysis of proteins isolated from AFFi-gel blue gel column chromatography of partially purified extracts from red kidney bean cotyledons (Affi-fraction). An aliquot (5 µl) of Bio-Rad prestained molecular weight standards and 12 µg of proteins from Affi-fraction were loaded, respectively, for SDS-PAGE. The labelled bands in the sample lane were excised and sent for MALDI MS/MS analysis.

4.3.4- Effect of commercial PHA-L on PPA activity

The commercial PHA-L exhibited a single band after SDS-PAGE (data not shown). It had no detectable amylase activity but was found to be also capable of promoting *in vitro* PPA activity (by about 80% with addition of 7 µg of purified PHA-L) when used instead of bean protein extracts in the α-AI assay using PPA (Fig. 4.8).

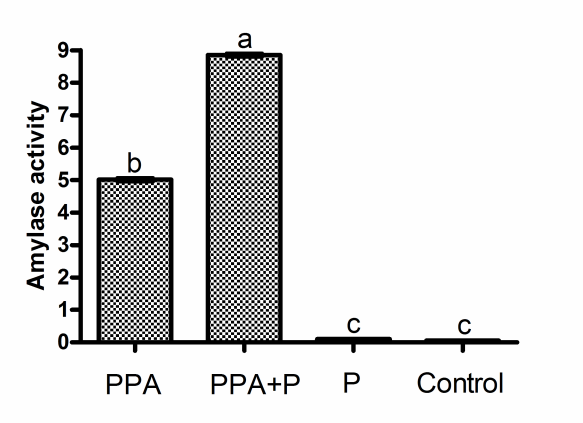

Figure 4.8- Enhancement of porcine pancreatic α-amylase activity (PPA) by a commercial PHA-L (7 μg protein). **P=** PHA-L. Control reaction was without PPA and Affi-fraction. Values labelled with the same letter do not differ significantly according to Duncan's multiple range test ($P<0.05$).

4.4- Discussion

All results in this chapter regarding α-amylase inhibitory activity determined based on the iodine-starch reaction were confirmed by comparing sugar release by amylase activity in the presence or absence of an inhibitory extract as well (data not shown). Presence of porcine pancreatic α-amylase inhibitor (α-AI) in red kidney bean seed extracts has been reported before (Grossi de Sa *et al*., 1997; Santimone *et al*., 2004) but assay of α-AI using extracts from the embryonic axis revealed an unexpected finding in that these extracts stimulated rather than inhibited porcine pancreatic α-amylase activity. PHA in red kidney bean seed has been shown to be an enhancer of PPA activity in a previous report (You and Chang, 1992). The stimulatory activity in the embryonic axis could also be attributed to PHA as the axis has been reported to contain PHA with the same properties as those of that isolated from the cotyledons (Greenwood *et al*., 1984). In-gel detection of α-AI after electrophoretic separation of proteins in extracts of the embryonic axis revealed the presence of at least 4 major isoinhibitors of porcine

pancreatic α-amylase. This suggested that the amylase inhibitory activity was masked by amylase stimulatory activity in the extracts of the axis. On the contrary, in extracts of the cotyledons amylase stimulatory activity was masked by amylase inhibitory activity. When cotyledonary extracts were used in the in-solution PPA inhibitor assay, the inhibitory activity was observed. It was, however, only after cotyledonary extracts had been placed in a boiling water bath (100 ± 2^0C) for 10 min resulting in the disappearance of most bands of isoinhibitor of PPA that the enhancing effect of the extracts on PPA in the in-solution PPA inhibitor assay was revealed.

α-Amylase inhibitory activity in the embryonic axis and cotyledons of common bean seeds (*Phaseolus vulgaris* cv. Greensleeves) has been detected by an immunoblotting technique in a previous study but no data based on in-solution assay was present regarding the presence of amylase inhibitory activity in crude extracts of either the seed coat or embryonic axis (Moreno *et al*., 1990). In the present study, no amylase isoinhibitor in extracts of red kidney bean seed coat was detected following non-denaturing polyacrylamide electrophoresis. But in-solution assays revealed that the seed coat extracts enhanced PPA activity. In a previous study, α- amylase inhibitor was also not detectable in extracts of common bean seed coat using an immunoblotting technique. This is similar to the present result but it had no data regarding PPA stimulatory activity in seed coat extract (Moreno et al., 1990). The reason for stimulatory activity in the seed coat can be investigated by some experiments to identify the stimulator. Based on prior studies PHA in *Phaseolus vulgaris* could be released from the root and from other seedling parts (Borrebaeck and Mattiasson, 1983; Kjemtrup et al.,

1995). Therefore, the PHA diffused to the seed coat could contribute to enhancement of PPA activity. More experiments are needed to confirm this.

PHA was implicated as a stimulator of PPA activity co-extracted with α-AI from the cotyledons following two independent protein purification procedures: using a single-step thyroglobulin column chromatography or sequential partial purification using DEAE-cellulose and Affi-gel blue gel columns followed by excising a protein band from SDS-PAGE gel for MALDI mass spectrometry. The excised protein band of apparent M_r of 31-33 KDa was identified as PHA-L, an extremely thermostable lectin (Biswas and Kayastha, 2002). The latter method further established that PHA-L is a stimulator of PPA activity which is consistent with the present finding that a purified commercial PHA-L was also shown to be capable of increasing PPA activity *in vitro*. Among the known applications of lectins and PHA in particular (You and Chang, 1992; Kordas *et al*., 2000; Rudiger and Gabius, 2001; Kunzelmann *et al*., 2004; Linderoth *et al*., 2006; Purhonen *et al*., 2008; Ren *et al*., 2008b), there is no result showing PHA-L to have an effect on PPA under *in vitro* conditions. If other unknown PPA stimulator proteins and PPA inhibitor proteins are present together in the same plant extracts, the identity of the stimulators in these extracts might only be revealed when purified proteins devoid of PPA inhibitory activity have been obtained. Furthermore, the present result also suggests that many studies might have underestimated the magnitude of PPA inhibitor activity. Particularly, in crude extracts there would be the potential interference of a PPA stimulator such as PHA-L present in the same extracts. Since detection and interpretation of assay results of inhibitor (α-AI) and stimulator (PHA) of

PPA activity in crude extracts could be complicated, it is probable that proteins with stimulatory effect on PPA have been overlooked in many previous studies.

The interaction of PHA-L as well as PHA might be specific with selected enzymes such as PPA since they exhibited no effect on α-amylases of *Aspergillus oryzae* and *Alternaria alternata*. Future work might include investigation of the effect of PHA on a large number of amylases from different sources to better evaluate the selectivity of PHA on amylase activities. In this research it was also found that porcine amylase stimulatory activity can be present in the different parts of red kidney bean seed.

There does not appear to be any prior study on red kidney bean extracts using starch-incorporated polyacrylamide gels for the analysis of electrophoretically separated isoinhibitors of PPA. However, using various other chromatographic methods or based on cDNA isolation and characterization, it has been generally accepted that there are two to four isoinhibitors of α-amylases in bean seed extracts. Only one of these major isoforms (particularly α-AI-1) has been shown to be active against PPA (Gibbs and Alli 1998; Santimone *et al*., 2004; Kluh *et al*., 2005; Obiro *et al*., 2008). Clearly, characterization of the various isoinhibitors of α-AI revealed in this study awaits further studies.

In enzyme studies and applications, it is most desirable to use enzyme activity as high as possible. The discovery of proteins such as PHA-L capable of enhancing *in vitro* enzyme activity is therefore important.

Low-molecular-weight non-protein organic substances such as polyethylene glycol (PEG) have also been shown to enhance PPA activity (Yoon and Robyt, 2005; Mukerjea *et al*., 2006). An important note that must be considered on PHA purification is avoidance of a protein concentration step using PEG. In preliminary experiments when PEG was used to remove excess water/buffer from protein extracts (enclosed within a dialysis membrane), it was found that PEG could pass through the membrane resulting in stimulation PPA activity. Freeze drying is recommended to remove additional water from the purified protein or last fraction rather than using PEG.

Polyethylene glycol (PEG 1500 Da) at 0.04% (w/v) has been shown to enhance *in vitro* porcine pancreatic and *Aspergillus oryzae* amylases activity by as much as 70 and 53% respectively (Yoon and Robyt, 2005). Interestingly, PHA was not able to increase *Aspergillus oryzae* amylases activity in the present study suggesting that PHA acted in a more specific manner in comparison with PEG. It has been hypothesized that PEG is likely involved in stabilizing the structure of the PPA (by means of binding to the enzyme) in a dilute solution leading to a higher enzyme activity (Yoon and Robyt, 2005). Later it was illustrated that amylase in the solution is present in several structural forms with different activities. Addition of PEG to the enzyme can cause a single structure formation which will lead to stabilization of the enzyme and finally enhancement of the activity (Mukerjea *et al*., 2006). The purified protein in the present study might also work in a similar way but this remains to be elucidated. Future studies into the interactions between PHA-L and other lectins at the protein-protein and

molecular level should shed new lights into the requirements for enhancement of PPA and probably other enzyme activities as well.

At least 8 min preincubation between PPA and an inhibitory extract has been found to be necessary for assay of PPA inhibitory activity in red kidney bean seed extract (chapter three). By contrast, only preincubation between PPA and a stimulatory extract for a couple of minutes was necessary for the assay of stimulatory activity. Although in the established assay PHA was preincubated with PPA before adding the substrate, it can be checked later if there is any detectable stimulation activity when PHA is preincubated with the substrate first. In a previous work no amylolytic activity enhancement was observed when PEG 1500 Da was added to the substrate alone. It was concluded that binding of the additive to the enzyme is important to get higher activity and stabilization. Increase in the amylase activity can be exclusively attributed to interaction between PEG and the enzyme but not because of the effect of PEG on the secondary or tertiary structure of the starch (Yoon and Robyt, 2005; Mukerjea *et al*., 2006). PEG was able to recover inactive enzyme (i.e. the enzyme which had been stood for 2 h under optimum condition) in a previous study (Yoon and Robyt, 2005). Therefore, it is worthwhile to investigate the effect of PHA on inactive PPA in future.
Interestingly, several animal glycoproteins including human transferrin and bovine fetuin (Matsushita *et al*., 2002) also exhibited an enhancing effect (in a dose-dependent manner in comparison to the control) on *in vitro* activity of PPA. It was hypothesized that at least in the case of the animal proteins tested PPA can bind to the N-linked sugar or oligosaccharides resulting in higher PPA activity (Matsushita *et al*., 2002). These proteins do not bind plant, salivary and fungal amylases and also have no effect on the activities

of these amylases. Conceivably, this might also be an explanation as to how PHA-L could enhance *in vitro* PPA activity. Similarly, PHA might interact specifically with selected enzymes such as PPA since it exhibits no effect on α-amylases of *Aspergillus oryzae* and *Alternaria alternata* (data not shown). Similar to the enhancing effect of PHA on *in vitro* PPA activity, a leaf lectin when added to insect gut homogenates was shown by Macedo *et al*., (2007) to enhance the insect gut α-amylase activity. Mechanism of action of the lectin was assumed by either increasing the number of active sites of the enzyme or by binding the enzyme to the substrate at somewhere different from their binding site. Therefore, it would seem possible that the occurrence of proteins in plants that can enhance animal α-amylase activities might be widespread and is worthy of further study. It would be also of interest to determine if plant proteins other than lectins possess an enhancing effect on PPA activity.

Several studies of feeding animals such as pigs and rats with PHA (the amount of PHA-L has not been specified) found some controversial effects on the secretion of pancreatic enzymes including α-amylase after hours and even several days of PHA administration (Grant *et al*., 1997; Herzig *et al*., 1997; Kordas *et al*., 2000; Baintner *et al*., 2004a and b; Linderoth *et al*., 2006; Thomsson *et al*., 2007). In the case of increased secretion of PPA following feeding PHA, it is not clear how long PHA could remain stable inside the animal body and particularly in the bodily fluids. It is unclear if the administered PHA might be directly responsible for an increase of PPA activity in the hog's pancreatic juice (Thomsson *et al*., 2007). It would be worthwhile to assess the effect of PHA on pancreatic amylase of rats in future.

Phytohemagglutinins have been reported to be resistant against proteolysis in the gut of rats (Kordas *et al*., 2000; Vasconcelos and Oliveira, 2004; Zhang *et al*., 2009a) while they were degraded up to 75% by bovine trypsin in the absence of Ca^{2+}. It was concluded that their resistance in the animal gut can be attributed to calcium content in common bean seed (Morari *et al*., 2008). It was found that phytohemagglutinins also possess bovine antitryptic activity (i.e. trypsin inhibitory activity not trypsin resistant) in the presence of Ca^{2+} (data not shown). PHAs can therefore be considered as multifunctional proteins.

Interestingly, an evolutionary intermediate was found between α-AI and another protein highly related to PHA (Wato *et al*., 2000). This molecular intermediate behaves like α-AI but is inactive against PPA. What is more intriguing is that purified PHA has stimulating PPA activity an unexpected property (You and Chang, 1992). Detection of the co-existence of PPA stimulator and inhibitor in the cotyledons, representing the bulk of food for animals, is not straightforward as the inhibitor activity seems to mask the effect of the stimulator. Therefore, it is a moot point that diametrically opposite biological functions (PPA stimulator and inhibitor) can reside in genes coding for PHA and α-AI, respectively that are evolutionarily so related. Molecular biological studies have uncovered a fascinating relationship between the α-amylase inhibitor (αAI) and phytohemagglutinins (PHA) found in bean seeds. They are from a single ancestral gene; their genes occur at the same locus in the bean genome and are very similar at the gene sequence level differing in only several amino acid residues. Overall α-AI was known as a truncated form of PHA (Chrispeels and Raikhel, 1991;

Mirkov *et al*., 1994; Hamelryck *et al*., 1996; Young *et al*., 1999; Wato *et al*., 2000). Both proteins are synthesized at the same time and stored in protein storage vacuoles (Moreno *et al*., 1990).

Other difference between α-amylase inhibitors (αAI) and phytohemagglutinins (PHA) is in their hemagglutinin activity. Four amino acids including an asparagine, an aspartate and two glycine residues are present in all lectins of legumes that are necessary in the carbohydrate-binding sites of the lectins. The middle residue is absent in α-amylase inhibitor making them devoid of hemagglutinating activity in contrast to phytohemagglutinins (Mirkov *et al*., 1994; Young *et al*., 1999; Ye *et al*., 2001b). As a linkage between the genes of αAI, PHA-E, PHA-L and arcelin has been found (Chrispeels and Raikhel, 1991), it would be of great interest to investigate if arcelin has a stimulatory activity on PPA.

Chapter 5- Studies on Conidiogenic Effect of Lectins

5.1- Introduction

Leguminous seeds are a good source of bioactive proteins (Reynoso-Camacho *et al*., 2006; Sridhar and Bhat, 2007). Examples of bioactive proteins are different plant lectins that can recognize and reversibly bind to specific sugars including mannose, galactose and N-acetylglucosamine of glycolipids, glycoproteins and other glycoconjugates on cell surfaces (Pusztai *et al*., 1993; Lei and Chang, 2009). While the physiological roles of plant lectins are still not clear (Van Damme *et al*., 2008), their effects on other organisms have been widely investigated (Vasconcelos and Oliveira 2004; Van Damme *et al*., 2008). In general, plant lectins may be antiviral, antibacterial, antifungal, anti-insect and even poisonous to higher animals including humans (Cowan, 1999; Ng 2004; Kaur *et al*., 2006; Singh *et al*., 2006; Keyaerts *et al*., 2007; Sitohy *et al*., 2007).

In contrast to these frequently-reported activities of plant lectins that are associated with biological defence in nature, a mannose-binding lectin (MBL) called PvFRIL (*Phaseolus vulgaris* fetal liver tyrosine kinase 3-receptor interacting lectin) isolated from red kidney bean seed was shown to be able to preserve the viability of human progenitor cells and prevent their differentiation and proliferation (Moore *et al*., 2000). Furthermore, the FRIL family proteins including the PvFRIL of red kidney bean and other legumes such as hyacinth bean (*Dolichos lablab*) may have additional medical

importance for treating inflammation-related diseases (Dinarello and Moore, 2006).

Spore formation in fungi has been reported as an energy-consuming process and may be induced by a variety of environmental factors including deprivation of nutrients such as nitrogen and carbon, illumination with light, osmotic stress, moisture stress, specific volatile organic chemicals, ozone and near ultra violet light (NUV) (Pascual *et al*., 1997; Masangkay *et al*., 2000; Yoshida and Shirata, 2000; Chovanec *et al*., 2001; Mills *et al*., 2004; Fischer and Kues, 2006; Carvalho *et al*., 2008; Nemcovic *et al*., 2008; Antony-Babu and Singleton, 2009; Zhang *et al*., 2009b). Some filamentous fungi produce conidia as dispersive propagules. Some fungal conidia can be used as biocontrol agents or biotransformation catalysts, commercial products in food industry and for antibiotic production (Smith and Calam 1980; Alexopoulos *et al*., 1996; Larroche and Gros 1997; Pascual *et al*., 1997; Jenkins *et al*., 1998; Park *et al*., 2000; Roncal and Ugalde 2003; Krasniewski *et al*., 2006; Xu *et al*., 2009; Zhang *et al*., 2009b). For instance, different strains of *Alternaria alternata* have been used as biocontrol agents against insects and *Plasmopara viticola*, a pathogen that causes downy mildew on grapevines (Hatzipapas *et al*., 2002; Musetti *et al*., 2006). Moreover, *A. alternata* f.sp. sphenocleae has been reported as a useful biological control agent for gooseweed (*Sphenoclea zeylanica*) (Masangkay *et al*., 2000). Spore production is also useful for identification and for experimentation but sometimes reliable conidiation on demand could be a problem. For example, study of *Alternaria alternata* from citrus has been problematic due to a poor rate of spore production and introducing new methods to induce spore formation could be useful in research progress

(Carvalho *et al*., 2008). Therefore, it is of great interest to discover new experimental means to induce fungal conidia formation *in vitro*.

To date, mulberry leaf extracts containing biotin have been shown to induce conidiation in a fungus (Yoshida and Shirata, 2000) and vitamin E as an antioxidant seems to have a negative effect on sporulation of *Aspergillus nidulans* (Emri *et al*., 2004). Some low molecular weight compounds either applied exogenously or occurring endogenously have also been reported to be involved in the regulation of conidia formation including a range of pheromones from terpenoid to derivatives of fatty acids (Fischer and Kues, 2006). However, it is generally not known if purified plant proteins have conidiation inducing activity. It could be a new area of research to assess the effect of bioactive proteins on conidiation.

Here we report investigations from an unexpected discovery that crude extracts of red kidney bean cotyledons exhibited such bioactive effect on *A. alternata* and *Aspergillus niger*. The new activity was found when a dark crescent was observed around the paper discs in antifungal experiments on PDA plates. Later, the protein with conidiogenic activity on *A. alternata* was purified and identified by MALDI mass spectrometry.

5.2- Materials and methods

5.2.1- Extraction of crude extracts from seeds

Cotyledons of red kidney bean (100g) were ground in 50 mM tris-HCl buffer (400 ml) at pH 8.0 containing 1 mM each of $CaCl_2$ and $MgCl_2$ and centrifuged at 10000×g for 10 min at 4^0C. The pH of the supernatant was

adjusted to 4.0 with concentrated HCl and after another round of centrifugation (10,000×g for 10 min), the supernatant (referred to as crude extract) was readjusted to pH 8.0 with 2 M NaOH. Initially the extract was assessed for either its conidiogenic activity or promotion of dark mycelia formation using the paper disc method as described in 2.6.1.

5.2.2- Protein identification

To identify the bean proteins possessing the previously unknown conidiogenic effect, crude extracts of bean cotyledons were processed through several protein purification steps as described in 4.2.2. The proteins in a purified fraction (Affi-fraction) still retaining a conidiogenic effect on *A. alternata* were separated by SDS-PAGE and stained with Coomassie Brilliant Blue (Fig. 4.7). Individual bands were cut from a gel after SDS-PAGE and sent to the Centre for Protein Research, Department of Biochemistry, Otago University (Dunedin, New Zealand) for MALDI tandem Time-of-Flight analysis (MALDI TOF/TOF, Applied Biosystemes, MA). The MS/MS data generated were searched against the UniProt/SWISS-PROT amino acid sequence database using the Mascot search engine (http://www.matrixscience.com).

5.2.3- Preparation of mannose-agarose column and purification of mannose-binding lectins

A mannose-agarose column (Cat. M6400, Sigma, St. Louis, USA) was used to purify mannose lectins from red kidney bean cotyledons according to Moore et al., 2000. It was found that mannose-agarose beads prepared using a method of Dr. Fabio Apone, Arterra Bioscience Srl, Via Benedetto Brin,

6980142, Napoli, Italy (personal communications) as described below, contained higher mannose content than commercially prepared ones and gave a better yield of bound protein. Therefore, a column packed with mannose-agarose prepared in the lab was used for purification of mannose lectins.

5.2.3.1-Preparation of adsorbent D-mannose Sepharose beads

Sepharose 6B (Cat. 6B100, Sigma, St. Louis, USA) beads (25 ml) was washed in a flask (250 ml) with distilled water for 4 times to eliminate salts and sodium azide before they were resuspended in 50 ml of 0.5 M carbonate buffer (0.5M Na_3CO_3 and 0.5M Na_2HCO_3, pH 11.0, BDH, Cat. 10240, Poole, England). To this suspension, 2.5 ml of divinyl-sulphone (DVS) (Cat. V3700, Sigma, St. Louis, USA) was added. The mixture was left at room temperature with gentle stirring for 90 min and then the beads were filtered and washed with distilled water through a glass filter funnel until the DVS was totally removed. The beads were resuspended in 25 ml of water and 25 ml of 20% D-mannose (Cat. M6020, Sigma) solution in 1 M carbonate buffer (the final concentration was 10% mannose in 0.5 M carbonate buffer) was added. The mixture was left at room temperature overnight on gentle shaking before the beads were then extensively washed with distilled water to remove excess sugar, resuspended in 50 ml of 0.5 M carbonate buffer and 0.5 ml of 2- mercaptoethanol (Cat. 441433A, BDH) was added. After incubation at room temperature for 2 h, the mixture was washed with distilled water to remove excess mercaptoethanol and sugar. Finally the beads were resuspended in 50 mM Tris-HCl buffer (50ml) pH 8.0 containing 1 mM each of $CaCl_2$ and $MgCl_2$ and used for purification of mannose-

binding lectin.

5.2.3.2- Purification of proteins

Extracts obtained as described in 5.2.1 were tumbled with mannose-agarose overnight at 4^0C and then transferred to a column (20 × 2.5 cm, Bio-Rad, Hercules, California, USA). The mannose-agarose beads were washed with TBS (Tris buffered saline comprised of 10 mM Tris and 150 mM NaCl at pH 7.5) to remove unadsorbed proteins and the bound mannose lectins were eluted using 0.2 M D-mannose. The eluted lectines were freeze-dried, redessolved in 10 mM Tris buffer (pH 7.3) and dialyzed against the same buffer at 4^0C overnight.

5.2.4- Evaluation of crude red kidney bean extracts and lectins on conidiogenesis of *Alternaria alternata*

A PDA (potato dextrose agar) disc method was developed in the lab to determine conidiogenic effect of lectins. Discs of PDA (1.5 cm diameter) were cut from a medium using a sterile test tube and transferred to a new sterile Petri plate (four discs in each Petri plate). The fungus (*Alternaria alternata*) was cultured on one side of PDA discs for 2 days at 26^0C. Then sterile paper strips (1×5 mm) were also placed on PDA blocks but 1 mm away from the growing margin of the fungal mycelium. To each paper strip, a different amount (0, 5, 10 or 20 µg) of lectins eluted from the mannose-agarose column or crude extracts containing 0, 5, 10 or 20 µg of soluble protein prepared from red kidney bean cotyledons was applied. After 24 h at 26^0C, the agar discs were transferred to 5 ml of washing solution containing 0.025% Tween 80 (BDH) and 0.8 M NaCl and then sonicated to isolate spores (Nemcovic *et al*., 2008). Spore counts were determined using a hemacytometer.

5.2.5- Spore germination assay

Spores obtained from different experimental treatments were suspended in sterile distilled water and spread on slides coated with a thin layer of PDA in a sterile Petri dish. The Petri dishes were incubated at 26^0C for 12 h and germinated spores were counted and also the length of germ tubes measured under a light microscope. Spores obtained from the fungus by light induction were used as control. At least 100 spores were considered for this assessment. Germ tube length measurements were carried out in the following order, one slide at a time: the conidia grown from cultures treated with MBLs, crude extract, from white light control and then this order was repeated again until all the slides were examined. The slides were kept at 4^0C until germ tube lengths were measured.

5.3- Results

5.3.1- Conidiogenic effect of mannose lectins isolated from red kidney bean cotyledons on *A. alternata*

In preliminary investigations, darkening of the periphery on the growing region of mycelia of *A. alternata* and *Aspergillus niger* as well as a growth inhibition crescent for the first fungus in the path of diffusing red kidney bean cotyledonary extract from a paper disc placed on the surface of a potato dextrose agar plate was observed (Figs. 5.1 and 5.2). Closer inspection of mycelium removed from the vicinity of the interface between the mycelium and a paper disc with crude red kidney bean extract under a light microscope showed the observed darkening was due to the induction of conidia at the mycelial periphery (Fig. 5.3). Crude extracts of red kidney bean cotyledons

were subsequently found to have conidiogenic effect on *Alternaria alternata* in a protein concentration-dependent manner (Fig. 5.4).

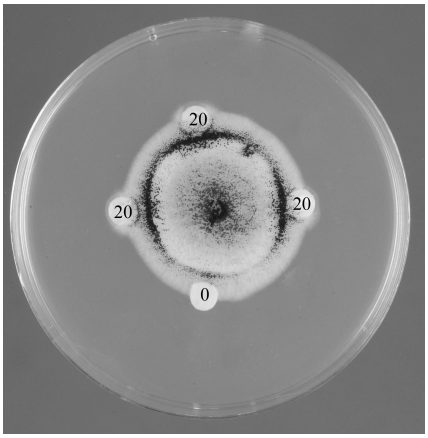

Figure 5.1- Effect of crude extract (20 µg protein) of red kidney bean cotyledons on *Aspergillus niger*. Dark periphery of the growing rim around the fungal colony due to melanization and conidiation was observed. Buffer (10 mM Tris-HCl) was used as control (0 µg protein).

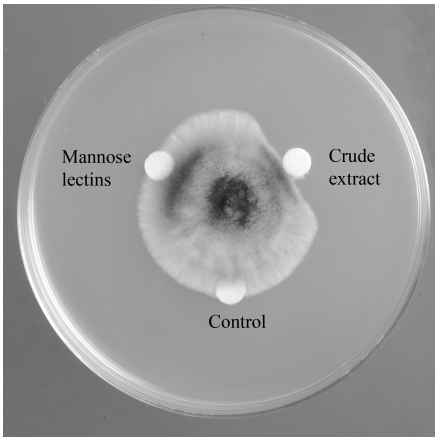

Figure 5.2- Effect on conidiation of crude extract or mannose-binding lectins, each with 20 µg of soluble proteins, prepared from red kidney bean cotyledons and applied to paper discs compared with buffer (10 mM Tris-HCl) as control.

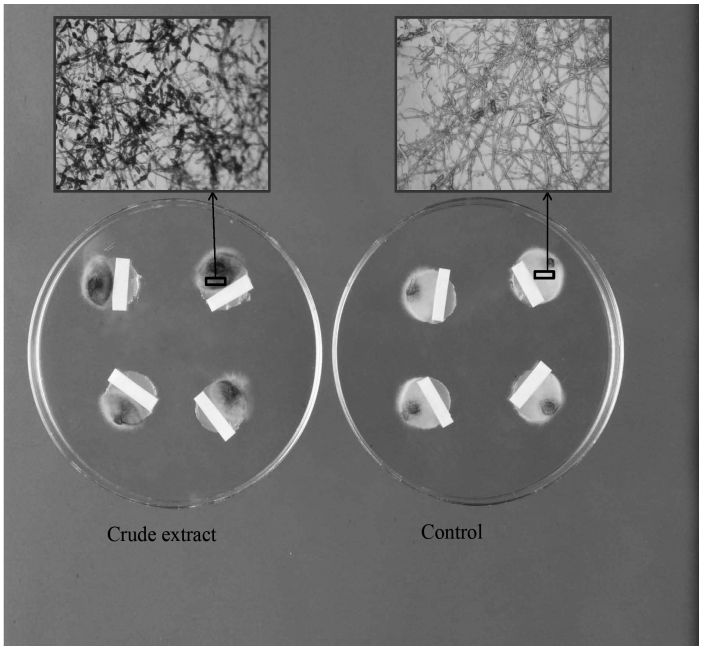

Figure 5.3- A PDA disc method to study conidiation of *Alternaria alternata*. Crude extract of red kidney bean cotyledons (20 μg soluble proteins) or buffer only as control applied to a paper strip was placed closed to the growing mycelium on the same agar disc. Following incubation in the dark at 26^0C overnight, mycelium in the vicinity of the paper strip was removed and viewed under a light microscope (shown in insets; magnification = 100×).

The respective bands (Affi-fraction, Fig. 4.7) were analysed using MALDI TOF/TOF, the 31 KDa polypeptide was most likely to be PHA-L (score 821) and those of the 14 and 21 KDa were most likely to be mannose lectins (scores 579 and 713 respectively and the mass spectra data shown in appendices). Interestingly, these two bands may be two truncated versions of the larger 31KDa mannose lectin protein. The band 2 (21 KDa on the gel) gave N-terminal fragment of the mannose lectin FRIL (residues 11-153) and band 3 (14 KDa) gave the C-terminal fragment (residues 135-254), with the centre fragment being present in both and so there may be overlapping fragments. It is possible that the denaturing conditions of the sample preparation for running the gel may be responsible for this or it could be that

the protein fragmented during purification process. In preliminary experiments, purified red kidney bean PHA-L (Sigma, Cat. No. L2769-2MG) had no conidiogenic effect on *A. alternata* (result not shown).

Subsequently, different amounts of mannose lectins eluted from mannose-agarose chromatography of crude extracts of red kidney bean cotyledons were confirmed to have, in a dose-dependent manner, conidiogenic effect on *A. alternata* compared to the control without addition of any bean protein (Fig. 5.4).

In another experiment, when crude extracts of red kidney bean cotyledons were applied to a mannose-agarose column the bound proteins eluted from the column were resolved on SDS-PAGE into three bands (Fig. 5.5).

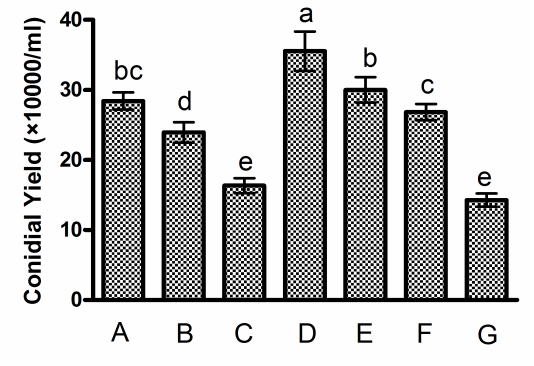

Figure 5.4- Effect of crude extract (**A**-20, **B**-10 and **C**-5 µg of soluble proteins) and mannose-binding lectins (**D**-20, **E**-10 and **F**-5 µg, isolated from red kidney bean cotyledons) and **G**, buffer on conidiation of *Alternaria alternata*. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

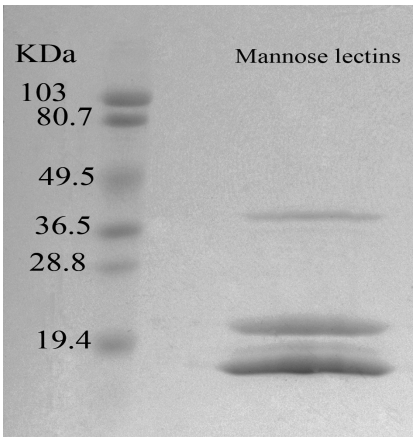

Figure 5.5- Mannose-binding lectins were purified from red kidney bean cotyledons by a mannose-agarose column and fractionated using SDS-PAGE with molecular mass standards (20 μg lectin).

A well characterized and commercially prepared MBL from Jack bean (*Canavalia ensiformis*), the concanavalin A type VI, used at five times the concentration of MBL from red kidney bean also had no CIE (result not shown). Another FRIL from hyacinth bean (kindly supplied by Dr. F. Apone, Aterra Bioscience, Italy) used at a similar concentration as PvFRIL had no CIE on *A. alternata* (unpublished results).

5.3.2- Conidium germination and germ tube length assays

The germination ability (% of conidia capable of producing germ tubes) of the conidia produced in response to white light control, addition of crude bean cotyledon extract or isolated bean mannose lectins was not significantly different (Fig. 5.6). However, germ tubes were significantly longer in those spores induced by the addition of 20 μg of mannose lectins isolated from red kidney bean cotyledons than those induced under white light control. Addition of crude extract containing 20 μg soluble proteins resulted in shorter germ tubes than the control (Fig. 5.7).

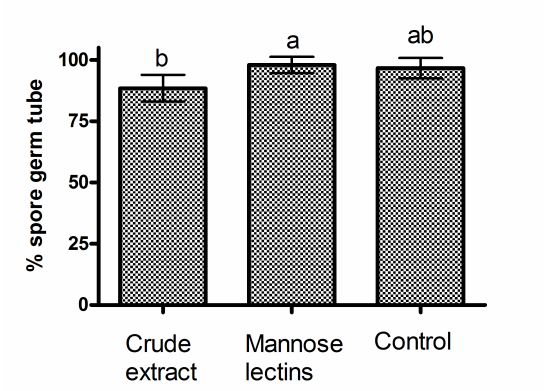

Figure 5.6- Germination of conidia formed in response to crude extract or mannose-binding lectins (20 µg) isolated from red kidney bean cotyledons or conidia from cultures grown under white light. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

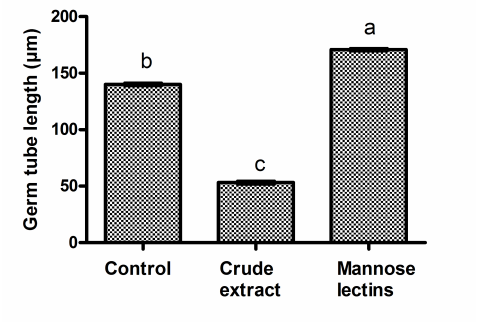

Figure 5.7- Length of germ tube (µm) of conidia produced in response to crude extract or mannose-binding lectins (20 µg) isolated from red kidney bean cotyledons. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

5.4- Discussion

All previous investigations about inducing factors of conidiogenesis have been limited to food sources, media, and light wavelengths (Xu *et al*., 2009). The darkening of a section of an *A*. *alternata* colony was first observed when cutting mycelial disks for subculture. Mycelial injury (wounding) has been reported before to induce conidiation in some fungi (Rodrigues *et al*., 2010). Later it was seen that darkening of mycelium occurred around paper

discs (Figs. 5.1 and 5.2) impregnated with the extract of red kidney bean seed. This resembled the central, older parts of colonies which also show conidial production. A crescent of melanization was present in colony of *Alternaria brassicae* in contact with an antifungal protein purified from *Psoralea corylifolia* (Yang *et al*., 2006) but surprisingly, the biological significance of this was apparently not recognised by other researchers.

The conventional PDA plate method of culturing fungal mycelium was useful for visual detection of conidiation (Fig. 5.2) but was not suitable for the purpose of quantitative determination of the number of conidia formed in response to application of a plant extract or protein solution. A variation of this method involving cutting discs from a colony on the PDA plate before transferring to a fresh Petri plate containing sterile paper strips impregnated with a test solution is also not useful to investigate induction of conidiation as wounding itself appeared to increase conidiation of *A. alternata*. Therefore, we used a small PDA disc (1.5 cm in diameter) which was essentially the mini version of the conventional PDA plate method to culture fungal mycelium to enable quantitative recovery and determination of conidia formed in the present study (Fig. 5.3).

Evidence obtained here strongly implicated a previously identified mannose-binding lectin, the PvFRIL, from red kidney bean seed to also possess conidiation inducing effect on a fungus. Therefore, it is suggested that PvFRIL can be a multifunctional protein. Proteins in the extracts were implicated to be responsible for the observed conidiation inducing effect

because overnight incubation with papain effectively abolished this effect (data not shown).

This is the first report on the effect of a protein isolated from another organism on conidiogenesis in fungi. Besides PvFRIL of red kidney bean, it would be of further interest to determine if other FRIL family proteins have similar conidiation inducing effect. It is possible that there could be proteins present apart from the mannose lectins that have a conidial inducing effect in *A. alternata* and other fungi. Exogenous application of a conidiation inducing protein is a new tool for induction of conidiogenesis. With this it might be possible to aid taxonomy of cryptic fungi, produce conidia on a large scale for fungal species that have medical importance or have biological control potentials for agricultural pests or weeds.

Interestingly, other lectins, including PHA of red kidney bean, FRIL of hyacinth bean and concanavalin A of Jack bean did not induce conidiation of *A. alternata*, suggesting specificity of the conidiogenesis inducing factor. Further studies are required to determine the range of fungi that mannose lectins from red kidney bean cotyledons can induce conidiogenesis.

The conidia formed in response to mannose lectin treatment did not differ from those induced under other conditions tested as far as spore germination was concerned. However, the length of germ tubes from MBL-induced conidia was significantly longer than those induced under other conditions. Germ tube length of MBL treatment was recorded at first and the remaining treatments were kept at 4^0C to prevent any likely growth during

measurement. For fast growing fungi, a photograph of germ tube length should be made initially so that the necessary measurements can be made later without complication. In the treatment with crude extracts of red kidney bean cotyledons, there might be an antifungal protein present together with MBL and the former inhibited elongation of germ tubes. In comparison with the white light treatment, the result suggested that mannose lectin-induced conidia were more vigorous as far as growth of germ tubes was concerned. The physiological basis for this difference is intriguing and worthy of further investigations.

The mechanism whereby applied PvFRIL can increase conidiation remains to be determined. It will also be of interest to determine whether this new approach to experimentally induce conidia formation involves similar or distinct underpinning cellular and molecular mechanisms compared to other conidia induction treatments. The molecular size of mannose lectins suggests that they might not need to be taken into hyphal cells for their conidiogenesis effect. This opens up a new avenue of research involving investigations into the biomolecular interactions between applied mannose lectins and the hyphal surface. Thus induction of conidiogenesis with mannose lectins would be a new and important approach to help gaining novel and further insights into conidiogenesis in fungi.

Transition from vegetative growth to conidiation of many fungi is often accompanied by an increase in production of secondary metabolites such as melanin which was likely to be at least partially responsible for the darkening appearance of the mycelium (Calvo *et al*., 2002). As shown in this

report, however it might be possible to uncover additional conidiation factors, including proteins that have been previously unrecognized in plant extracts.

It is worth noting that addition of crude extracts from many plants to the edge of a growing mycelium might cause a growth inhibition crescent which may be associated with darkening of the peripheral region of the mycelium. This darkening might be due to increased melanin accumulation and/or conidiogenesis. This might have complicated previous studies aiming to uncover the occurrence of any conidiation inducing factor in crude plant extracts. Closer inspection under a light microscope eventually led to the present investigations culminating in the conclusion that the induced darkening of the mycelial periphery was due to conidia formation and not purely increased melanin formation. It might be important to re-examine other reports that showed darkening mycelial periphery following addition of extracts from other plants. It might be possible to reveal additional conidiogenesis inducing factors including proteins that could have been previously unrecognized in diverse plant extracts.

Chapter 6- Changes in Biological Activities During Seed Development

6.1- Introduction

Leguminous seeds consist of many bioactive proteins including a variety of proteinaceous inhibitors and hydrolytic enzymes (Gomes *et al*., 1996; Santimone *et al*., 2004; Guillamon *et al*., 2008). These proteins accumulated in seeds during seed development and may play a role in seed defence against pathogen invasion during germination (Swegle *et al*., 1992; Gomes *et al*., 1996; Harsulkar *et al*., 1997; Sotelo and Lucas, 1998; Lima *et al*., 2002; Leubner-Metzger, 2003; Sekeli *et al*., 2003; Kokiladevi *et al*., 2005; Kumar *et al*., 2005; Guillamon *et al*., 2008). Some of these proteins might have other functional roles in plants. For example, chitinases have been reported to be associated with development of plant embryo and sexual reproduction (Leung, 1992; Lima *et al*., 2002). Most of the previous studies on developmental changes of bioactivities have focused on the extracts from whole legume seeds. Relatively fewer studies had been carried out to determine the changes in bioactivities in the extracts of pods or seed parts (Carasco and Xavier-Filho, 1981; Tadera *et al*., 1984; Mauch *et al*., 1988; Sotelo and Lucas, 1998; Kumar *et al*., 2006). In *Phaseolus vulgaris* cv. Jampa the changes in trypsin inhibitory activity were assessed at different stages of seed development (including flowers, string bean and ripe bean) (Sotelo and Lucas, 1998). No activity was found in either string bean or flower but it was detected in ripe and dry seeds. More comprehensive

investigation could include activity determination at different stages of development in different seed parts and pods. There is a lack of information about changes in amylase inhibitors, β-1, 3- glucanase and chitinase activities in red kidney bean seed parts and pods during seed formation. However, there are some studies on changes in these bioactive proteins during seed development of other legumes (Harsulkar et al., 1997; Kokiladevi *et al*., 2005; Kumar *et al*., 2005). In this chapter developmental changes of the selected bioactive proteins (porcine α-amylase inhibitor/stimulator, bovine trypsin inhibitors, β-1, 3- glucanase and chitinase activities) in red kidney bean seed parts (seed coat, embryonic axis and cotyledons) were evaluated during seed formation under glasshouse conditions. Changes in the activities of the selected bioactive proteins in the extracts of the pods during seed development were also investigated. In addition, a new simplified electrophoretic in-gel detection of bovine trypsin isoinhibitors present in the seed parts was developed. This new protocol takes advantage of a readily available coloured protein, azoalbumin, which has been used satisfactorily as a substrate for assay of endopeptidases (Mahagamasekera and Leung, 2001). It eliminates both the staining and destaining requirements as specified in other previously published protocols.

6.2- Materials and methods

6.2.1- Preparation of bean seed extracts from developing seeds

Red kidney bean seeds were germinated in distilled water in the lab and then sown in large pots (2.5 L) of soil for growing in the glasshouse at the University of Canterbury. Day and night temperatures were between 20-30^{0}C and 15-20^{0}C respectively. Flowers formed were tagged at anthesis.

Seeds and pods were harvested at 10 days from the time of flower fall and at 5 day intervals until seeds were fully matured (or when the seed pods were visibly desiccated) (Kumar *et al*., 2005). After harvest, seeds and pods were stored at -80^{0}C until they were analysed for the biochemical activities of interest in this study and antifungal activity against *A. alternata*.

Figure 6.1- Growing red kidney been seedlings in the glasshouse of University of Canterbury.

Figure 6.2- A tagged flower of red kidney bean.

6.2.2- Extraction and assays of biochemical activities

The seed parts and pods were separated, extracted as described in 2.2 and then their antifungal and biochemical activities (porcine α-amylase inhibitor/stimulator, bovine trypsin inhibitor, β-1, 3- glucanase and chitinase) were assessed as described in 2.6 and 2.7. Ten and 15-day old seeds were too small to be separated into seed parts. Moreover, the seed parts including the seed coat, embryonic axis and cotyledons were found to adhere together tightly at these ages that made it difficult to separate them apart. Therefore, whole red kidney bean seeds harvested at these two time points were used in this project to prepare extracts for evaluation of their bioactive protein contents, while those harvested at subsequent times were separated into the different seed parts and extracted as described in 2.2. Isoinhibitors of bovine trypsin present in red kidney bean seed parts were detected as described in 2.5.2.2. Red kidney bean seed parts collected during development were analysed for their water content as described in 2.3.

6.3- Results

6.3.1- Harvesting seeds and investigations into changes in biological activities during seed development

6.3.1.1- Visible changes in seeds and pods

The size of red kidney bean seed increased more than four fold from 10 days after flower fall to maturation (data not shown). Seeds were green up to 20 days after flower fall and then started changing to reddish (Fig. 6.3). No visible fibers were found on pods up to 15 days after flower fall but they appeared at day 20.

Figure 6.3- Seed development in red kidney bean. From left to right; seeds and pods at 10, 15, 20, 25, 30, 35, 45 days after flower fall and at the mature dried seed stge.

The percentage of water in developing seeds, pods and seed parts was calculated and the mean values were separated using Duncan's Multiple Range Test ($P<0.05\%$) (Table 6.1). Overall, their water contents increased up to 15 days after flower fall and then started decreasing significantly. Minimum water contents in seed, seed parts and pods were found at day 50 (the seed maturation stage).

Table 6.1- The percentage of water in developing seeds, pods and seed parts of red kidney bean. Means in each row labelled with the same letter do not differ significantly according to Duncan's MRT (P<0.05).

Seed Parts & pod	%water 10d	%water 15d	%water 20d	%water 25d	%water 30d	%water 35d	%water 40d	%water 45d	%water 50d
Whole seed	81.57 ±0.3**b**	85.73 ±0.35**a**	80.59 ±0.26**c**	73.68 ±0.46**d**	63.92 ±0.40**e**	59.73 ±0.65**f**	48.18 ±0.34**g**	35.21 ±0.45**h**	11.08 ±0.39**i**
Cotyledon	N/A	N/A	81.14 ±0.60**a**	71.19 ±0.60**b**	60.01 ±0.18**c**	55.07 ±0.66**d**	44.65 ±0.60**e**	31.49 ±0.66**f**	11.38 ±0.46**g**
Embryonic axis	N/A	N/A	78.1 ±0.42**a**	71.15 ±0.79**b**	65.56 ±0.59**c**	63.9 ±0.47**d**	53.85 ±0.64**e**	41.32 ±0.77**f**	9.46 ±0.28**g**
Seed Coat	N/A	N/A	79.61 ±0.56**a**	79.7 ±0.55**a**	77.47 ±0.55**b**	76.23 ±0.75**c**	64.42 ±0.71**d**	52.85 ±0.87**e**	7.52 ±0.44**f**
Pod	90.19 ±0.442**b**	92.52 ±0.58**a**	90.54 ±0.68**b**	88.49 ±0.60**c**	86.96 ±0.66**d**	85.28 ±0.58**e**	74.13 ±0.74**f**	66.92 ±0.46**g**	8.44 ±0.49**h**

6.3.1.2- Changes in biochemical activities in seed parts and pods during seed formation

6.3.1.2.1- Changes in AF (antifungal) activity during seed formation

No antifungal activity was detected in the seed coat and pod at any stage of seed formation. Extracts of immature whole seeds collected at 10 and 15 days after flower fall also did not show AF activity (data not shown). Before day 20, there was no AF activity in the extracts of either cotyledons or embryonic axis. At day 25, AF activity appeared in the cotyledons and increased significantly at day 30 (Fig. 6.4). In the embryonic axis, AF activity appeared at day 30 and increased afterwards at day 35 before remaining at a constant level until seed maturation (Fig. 6.6). There were no significant changes in the levels of AF activity from day 30 until seed maturation in cotyledon extracts.

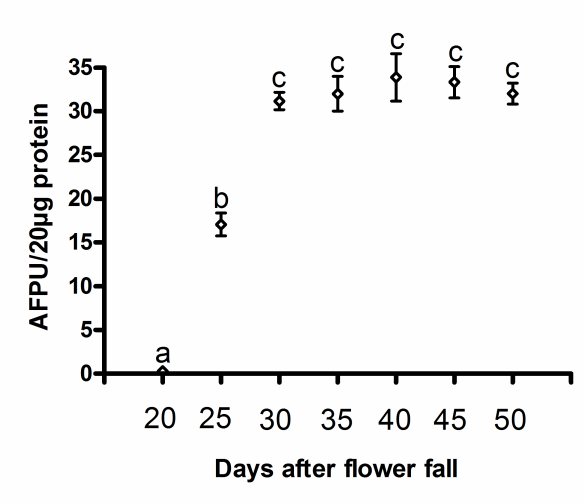

Figure 6.4- Changes in AF activity in red kidney bean cotyledons during seed formation. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$). Seeds were harvested at 20, 25, 30, 35, 40, 45 and 50 days after flower fall.

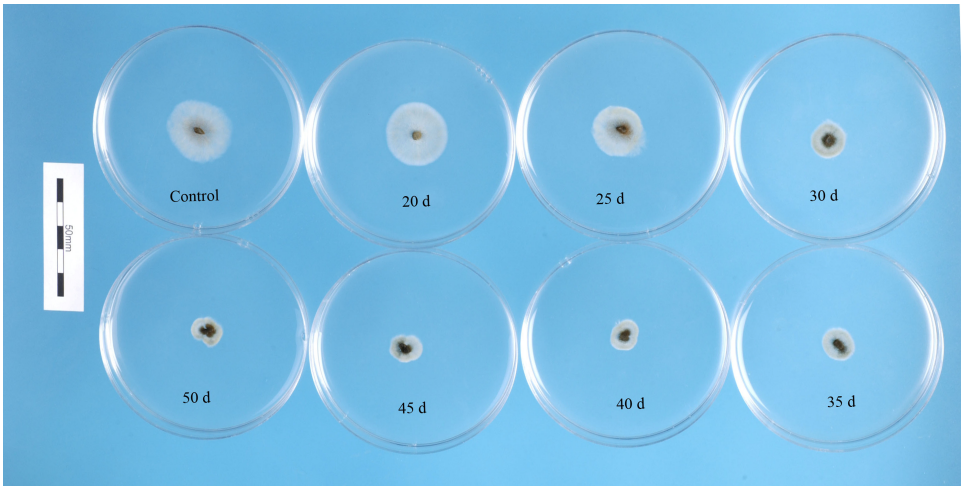

Figure 6.5- Changes in the area of *Alternaria alternata* colonies in the presence of antifungal protein isolated from red kidney bean cotyledons at various times during seed formation.

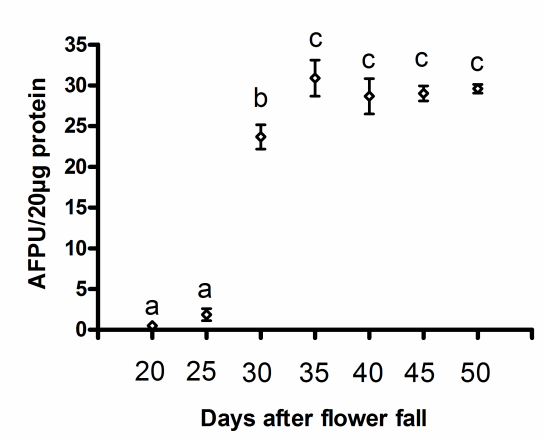

Figure 6.6- Changes in AF activity in red kidney bean embryonic axes during seed formation. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$). Seeds were harvested at 20, 25, 30, 35, 40, 45 and 50 days after flower fall.

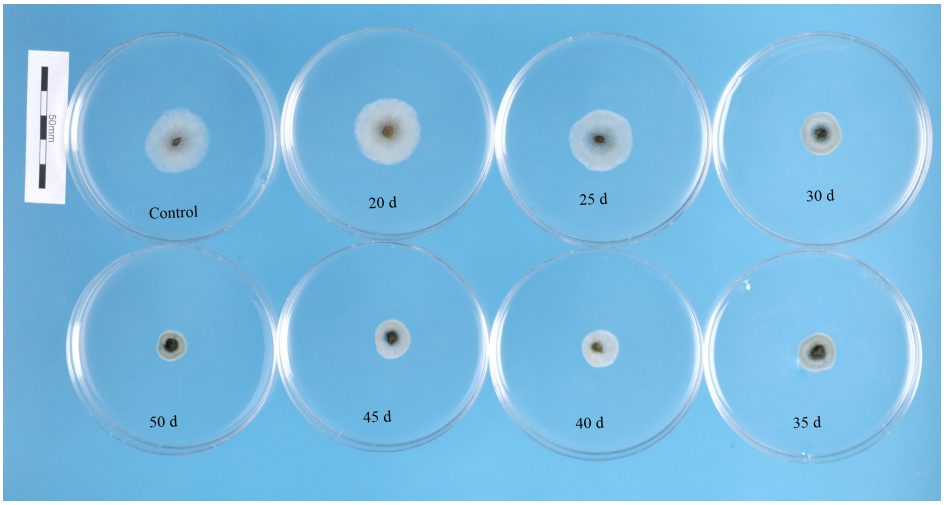

Figure 6.7- Changes in the area of *Alternaria alternata* colonies in the presence of antifungal protein isolated from red kidney bean embryonic axis at various times during seed formation.

6.3.1.2.2- Changes in bovine trypsin inhibitory activity during seed formation

Specific activity of bovine trypsin inhibitor was detectable in extracts of immature red kidney bean seeds at day 10 after flower fall and it increased significantly by day 15 (Fig. 6.8A). Specific activity of bovine trypsin

inhibitory was also found in extracts of red kidney bean cotyledons at day 20 after flower fall. It then increased with a transient peak at day 30 followed by a lower but constant level over the next 15 days. There was a further and significant drop in specific trypsin inhibitory activity by day 50 (Fig. 6.8B). In the embryonic axis specific trypsin inhibitory activity increased from day 20 and peaked at day 35 and 40. Then it continued to decrease (Fig. 6.8C). The level of specific trypsin inhibitory activity in the seed coat at day 20 was relatively high compared to that in the cotyledons and embryonic axis at the same time (compare Figs. 6.8B, C and D). Then specific trypsin inhibitory activity increased to peak levels at day 25 and 30 before it decreased (Fig. 6.8D). In pods, specific trypsin inhibitory activity increased from day 10 after flower fall and reached peak levels from day 20 to day 30. Then it started to decline until it was not detectable in pods at the seed maturation stage Fig. 6.8E).

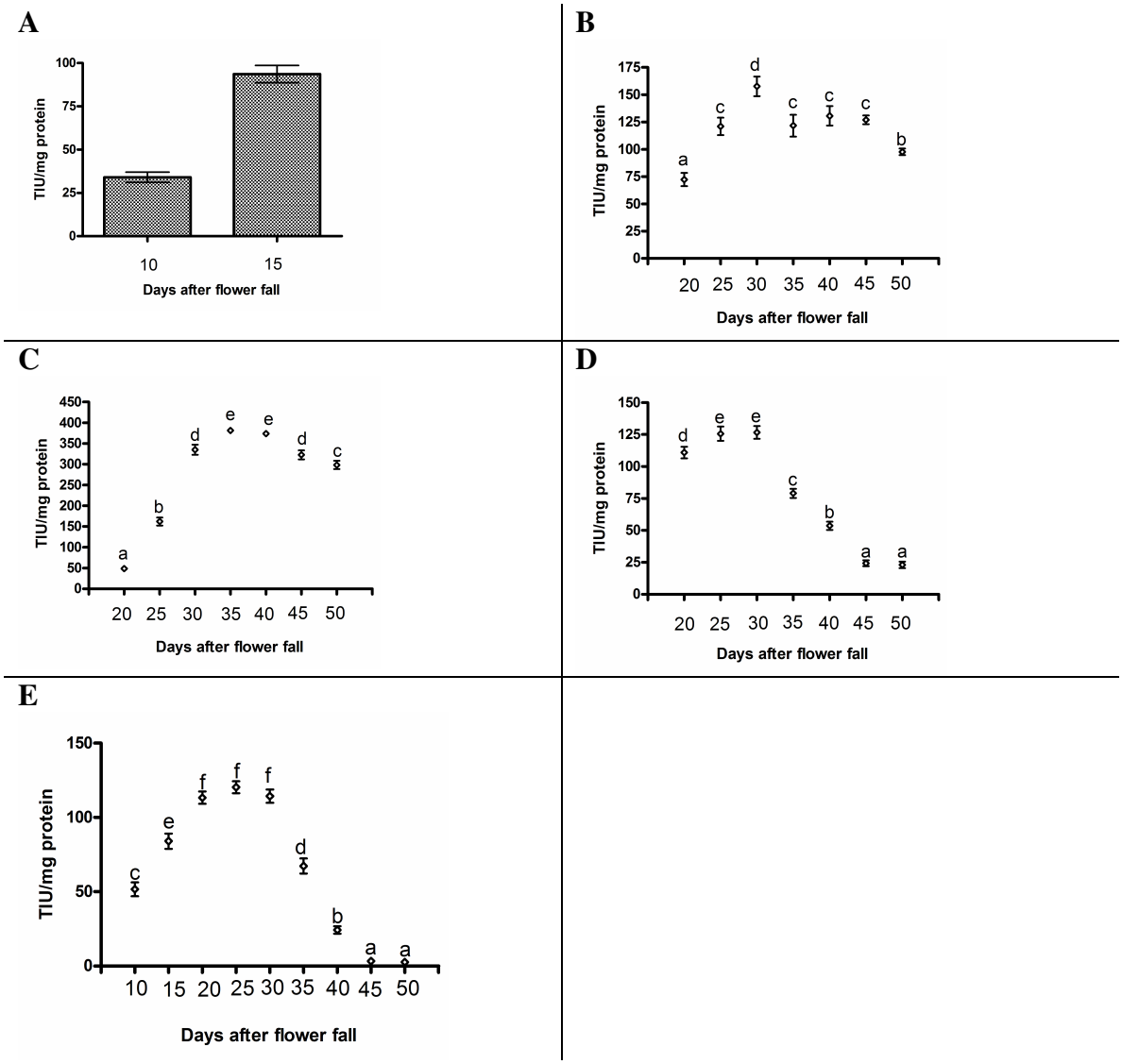

Figure 6.8- Changes in specific activity of bovine trypsin inhibitor in red kidney bean pods and seed parts during seed formation. **A-** Immature seeds, **B-** Cotyledons, **C-** Embryonic axes, **D-** Seed coats and **E-** Pods. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

In preliminary experiments, using different amounts (1 to 15 µg) of soybean trypsin inhibitor as a positive control for in-gel detection of trypsin inhibitor, it was found that 5 µg of the inhibitor was best resolved under the present conditions and 10 µg or more resulted in overloading and very poor

resolution. There was a good contrast between the yellowish background in the area of the gel without trypsin inhibitor and reddish-orange undigested azoalbumin bands in the gel indicating the presence of trypsin inhibitors therein (Fig. 6.9).

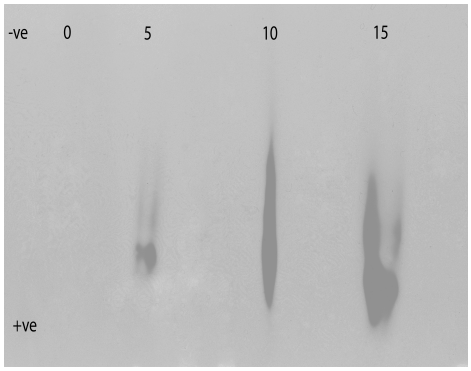

Figure 6.9- Azoalbumin-PAGE of different amounts (0-15 μg of proteins) of soybean trypsin inhibitor (Sigma catalogue no. T6522).

After electrophoretic separation of different extracts, incubation of bovine trypsin with non-denaturing polyacrylamide gel containing azoalbumin was successful in revealing the trypsin isoinhibitor profiles of the cotyledons and embryonic axis extracts (Fig. 6.10). The red kidney bean cotyledons and embryonic axis appeared to have two similar trypsin isoinhibitors in non-denaturing polyacrylamide gels with immobilized azoalbumin as far as relative electrophoretic mobilities were concerned.

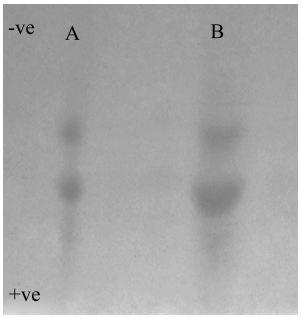

Figure 6.10 - Azoalbumin-PAGE of trypsin inhibitors. Each lane was loaded with a crude extract containing 10 µg proteins. **A=** Cotyledon extract of red kidney bean, **B=** embryonic axis extract of red kidney bean.

6.3.1.2.3- Changes in porcine α-amylase inhibitory/stimulatory activity during seed formation

Interestingly the same level of porcine amylase stimulatory activity was found in extracts of immature whole red kidney bean seeds harvested at day 10 and 15 after flower fall (Fig. 6.11). No porcine amylase isoinhibitor band was detected when whole proteins of bean seed were separated on non-denaturing polyacrylamide gels. So isoinhibitors of PPA had not been produced at this stage (data not shown).

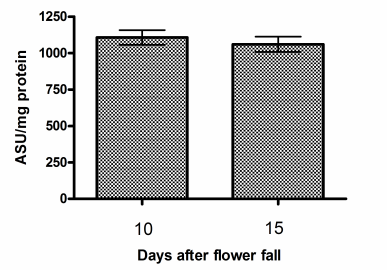

Figure 6.11- Changes in specific activity of α-amylase stimulator in extracts of immature whole red kidney bean seeds during seed formation. Seeds were harvested at 10 and 15 days after flower fall.

The specific activity of amylase inhibitor increased from day 20 after flower fall in red kidney bean cotyledons until day 30. Then this level was

maintained till day 45when a further increase occurred at seed maturation. No stimulatory activity was detectable in extracts of the cotyledons during seed development (Fig. 6.12).

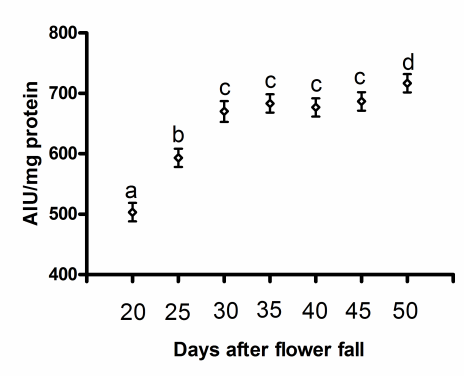

Figure 6.12- Changes in specific activity of α-amylase inhibitor in red kidney bean cotyledons during seed formation. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

No inhibitory activity was detected in extracts of embryonic axis using test tube tests by either sugar assay or starch-iodine complex method (Fig. 6.13). When extracts of the embryonic axis were separated on non-denaturing polyacrylamide gels, isoinhibitors of porcine amylase clearly were present at day 25 after flower fall (Fig. 6.14).

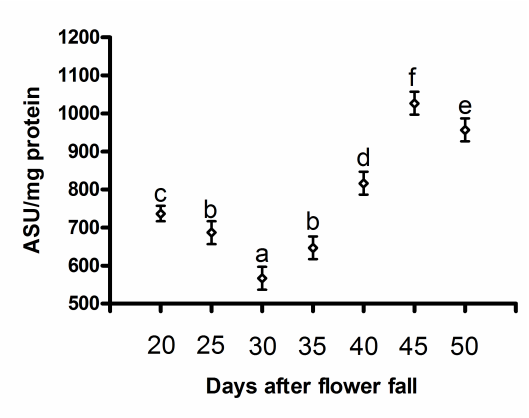

Figure 6.13- Changes in specific activity of α-amylase stimulator in red kidney bean embryonic axes during seed formation. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

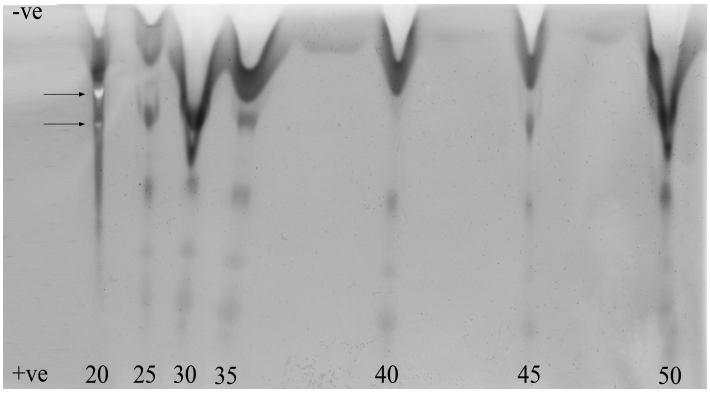

Figure 6.14- Detection of isoinhibitors of PPA activity following non-denaturing PAGE of extracts from the embryonic axis of red kidney bean seeds during seed formation. Equal amount of protein (10 µg) was loaded in each lane. At day 20 clear bands can be observed that can be attributed to stimulators (see arrows).

Extracts of the seed coat from immature red kidney bean seeds were able to enhance PPA activity. The specific activity of PPA stimulator decreased from day 20 till day 30 before in increased transiently at day 35. Then it decreased till day 45 before increasing at day 50 (Fig. 6.15). No isoinhibitor band was found on non-denaturing polyacrylamide gels used for

electrophoretic separation of proteins in extracts of the seed coat from immature red kidney bean seeds (data not shown).

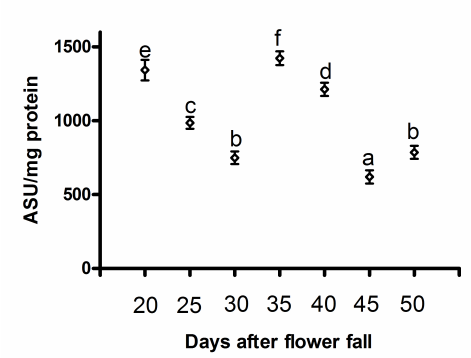

Figure 6.15- Changes in specific activity of α-amylase stimulator in red kidney bean seed coats during seed formation. Means labeled with the same letter do not differ significantly according to Duncan's MRT (P<0.05).

The PPA stimulatory activity was detectable in pods as well. There were high levels of the stimulatory activity in pods until 30 days. Then the PPA stimulatory activity started to decline greatly. It became non-detectable from day 45 (Fig. 6.16). No isoinhibitor band was found on non-denaturing polyacrylamide gels used for electrophoretic separation of proteins in the extracts of pods from immature red kidney bean seeds (data not shown).

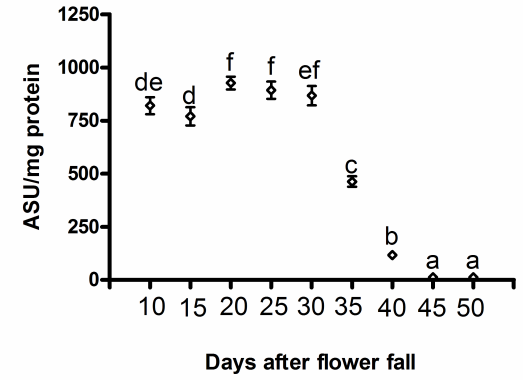

Figure 6.16- Changes in specific activity of α-amylase stimulator in red kidney bean pods during seed formation. Means labeled with the same letter do not differ significantly according to Duncan's MRT (P<0.05).

6.3.1.2.4- Changes in chitinase activity during seed formation

Chitinase activity was present in immature whole red kidney bean seed 10 days after flower fall and increased significantly at day 15 (Fig. 6.17A). Specific chitinase activity increased in the cotyledons from day 20 until day 45 before it decreased (Fig. 6.17B). Specific chitinase activity in extracts of the embryonic axis increased during seed development. The maximum activity was observed at day 45 before it decreased (Fig. 6.17C). The enzyme activity in the seed coat was highest at day 25 after flower fall. Then it decreased to low levels at seed maturation (Fig. 6.17D). In the pod the enzyme activity increased until day 30. Then it started to decline continuously and it was not detectable at day 45 (Fig. 6.17E).

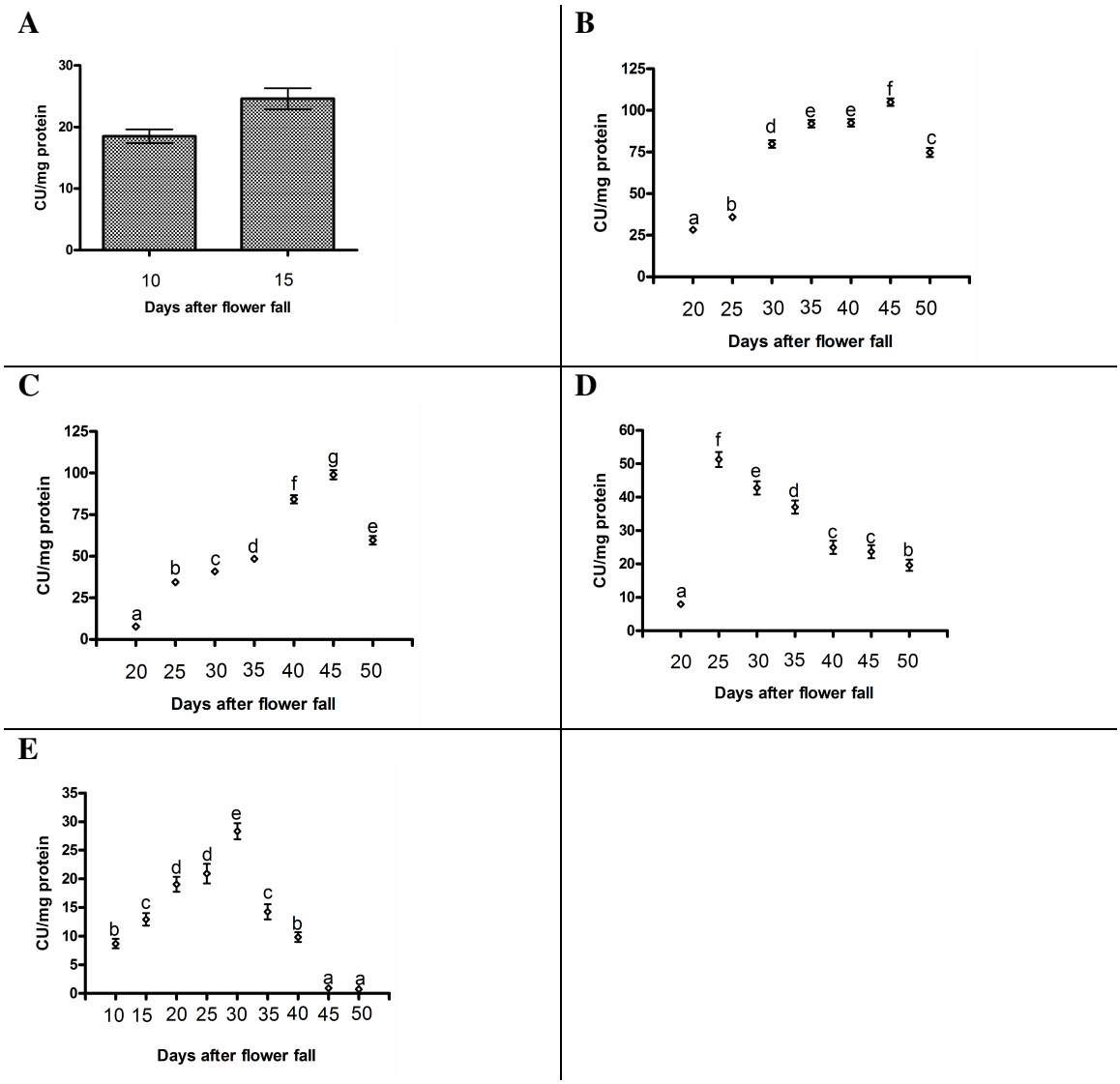

Figure 6.17- Changes in specific activity of chitinase in red kidney bean pods and seed parts during seed formation. **A-** Immature seeds, **B-** Cotyledons, **C-** Embryonic axes, **D-** Seed coats and **E-** Pods. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

6.3.1.2.5- Changes in β-1, 3- glucanase activity during seed formation

Specific activity of β-1, 3- glucanase in immature whole red kidney bean seed was significantly higher at day 15 than at day 10 (Fig. 6.18A). The enzyme activity in the cotyledons was not significantly different up to 25 days after flower fall. Then it increased until day 45 (Fig. 6.18B). In the embryonic axis the enzyme activity increased until day 40 after flower fall.

Then there was no further increase in the next 5 days before it decreased slightly at day 50 (Fig. 6.18C). Increase of the enzyme activity in the seed coat peaked at day 35 and 40 before it started to decline to the same level as that at day 20 (Fig. 6.18D). The changes in the enzyme activity in the pods were similar to those in the seed coat (Fig. 6.18E). At seed maturation the enzyme activity was undetectable.

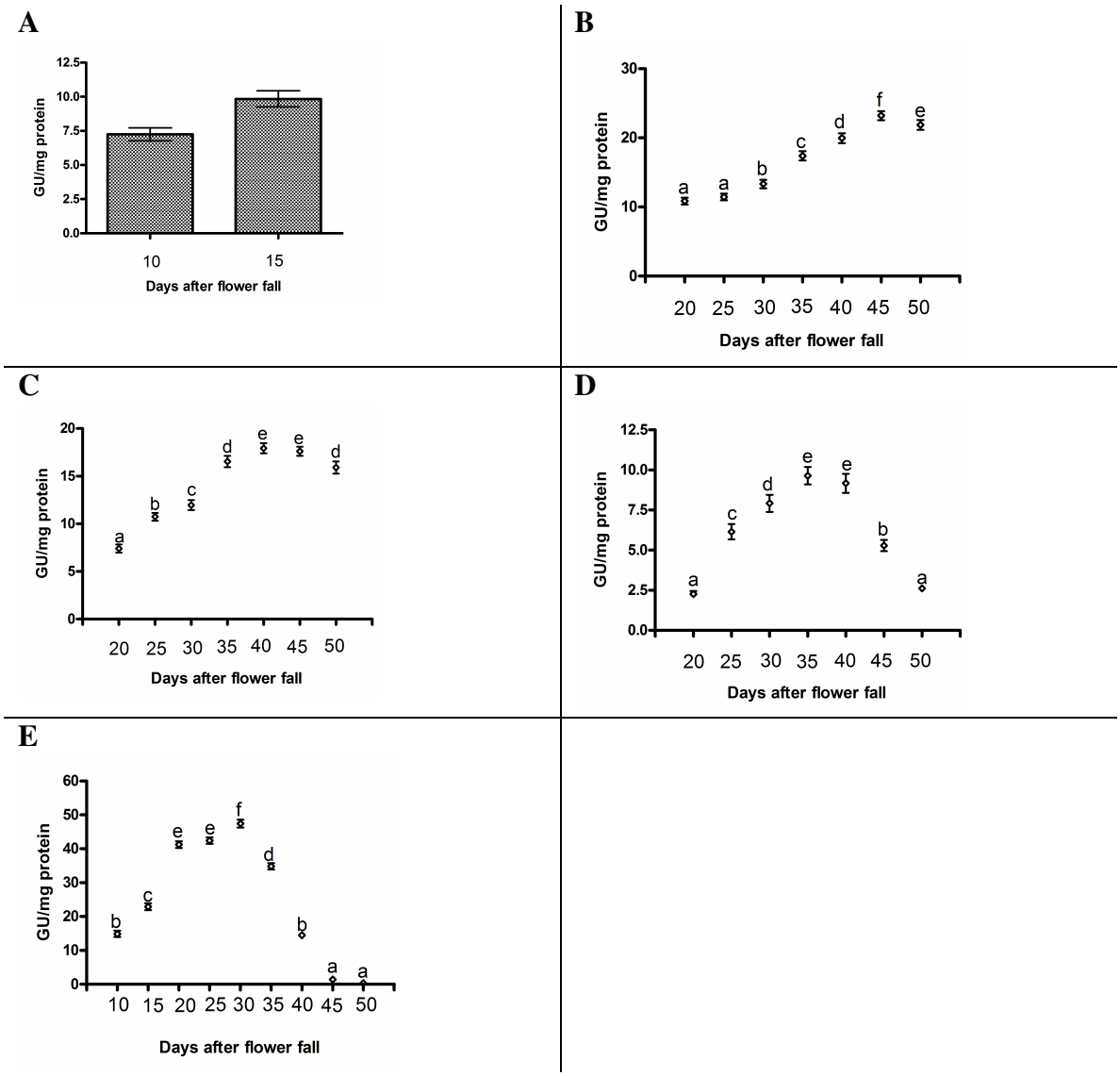

Figure 6.18- Changes in specific activity of β-1, 3- glucanase in red kidney bean pods and seed parts during seed formation. **A-** Immature seeds, **B-** Cotyledons, **C-** Embryonic axes, **D-** Seed coats and **E-** Pods. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

6.4- Discussion

Occurrence of AFPs at different developmental stages is thought to be related to plant's ability to defend against pathogens (Lima *et al*., 2002). Extracts of the seed coat and pod of red kidney bean did not show antifungal activity while those of the cotyledons and embryonic axis did. Increase of

AF activity in the cotyledons started from 20 days after flower fall while that in the embryonic axis started 5 days later. The significance of the observed developmental changes and localization of AF activity during seed development of red kidney bean is interesting but is not clear. It would be useful to elucidate this further if the accompanying changes in AFPs are also assessed during seed development in future studies using some methods such as Western blotting or gene expression quantification.

Bovine trypsin inhibitory (TI) activity was observed in all seed parts as well as the pod of red kidney bean. These results confirm the findings of previous studies (Fernandez *et al*., 1982; Texier *et al*., 1989; Domash *et al*., 2006). Presence of trypsin inhibitors have been shown in soybean (Santos *et al*., 2008) but not cowpea seed coat (Carasco and Xavier-Filho, 1081), pods of common bean and winged bean (Tadera *et al*., 1984; Texier *et al*., 1989).

The highest specific TI activity in the cotyledons and embryonic axis of red kidney bean was detected at day 30 and day 35 after flower fall, respectively. This was similar to the development of AF activity. TI activity in the seed coat at seed maturation was the lowest in comparison with that at the other seed developmental stages. Also, the seed coat was darker, probably due to increased tannin accumulation therein. This might protect the mature seeds besides the low level of TI activity against predators and pathogens. Future work can focus on isolation and purification of bovine trypsin inhibitors from pods and seed parts to see whether they are the same or not.

In preliminary experiments to develop an appropriate in-gel detection of trypsin isoinhibitors after electrophoretic separation of proteins in seed extracts it was found that azoalbumin concentration lower than 3% (w/v) in the gels did not yield any useful data regarding the trypsin inhibitor profile. Therefore, an important factor in achieving a clear distinction between the background and the area of azoalbumin in the gels that is protected from trypsin action is the percentage of azoalbumin used for its immobilization in gels. There are other procedures such as SDS-PAGE followed by renaturation with TritonX-100 and 2-D gel electrophoresis with higher sensitivity and greater resolution power coupled with protein identification techniques (Alves *et al*., 2010). The present method, however, is more appropriate for physiological studies working with numerous crude extracts or studies requiring rapid phytochemical analysis including trypsin isoinhibitor content of different breeding materials/lines and rapid evaluation expression of appropriate cDNA clones. The existing trypsin isoinhibitor gel detection methods are more time-consuming (several hours to overnight incubation) and laborious because of the need for protein staining followed by destaining steps as well. The commercially available Blue Casein gel system (Muzquiz *et al*., 2004) still requires a laborious renaturation step, unlike the present protocol using azoalbumin. This simple protocol could be readily adapted for detection of other types of protease inhibitors such as papain-like inhibitors. The ease of setting up the gels with azoalbumin and the relatively low cost to achieve this level of simplicity in detection of protease inhibitor profile without prior purification of biological extracts should be of great help in diverse research fields. Additionally, this simple procedure of protease inhibitor detection in polyacrylamide gels can also be adapted for use in a laboratory without a fumehood or an undergraduate

biochemistry laboratory class as the students would not need to use hazardous/ unpleasant organic solutions such as methanol and acetic acid required for protein staining and destaining in other methods for post-electrophoresis detection of protease inhibitors.

In a previous method, for detection of trypsin isoinhibitors after electrophoresis of an extract, the proteins in a gel have to be degraded by added trypsin. Presence of trypsin inhibitors is revealed after staining and destaining the gel using Coomassie Brilliant Blue (Alves *et al*., 2010). A question arises as to whether after Coomassie Brilliant Blue staining visible bands on a gel are truly trypsin inhibitors. Is it possible that some of them are just resistant to digestion by trypsin but unable to inhibit digestion of other protein substrates by trypsin? In the immobilized azoalbumin method developed here, trypsin inhibitor protein activity is defined as that of a protein that inhibits trypsin to digest another protein (azoalbumin). Alternatively, it is necessary to purify proteins and then confirm their trypsin inhibitory activity in test tubes as well. The finding that PvFRIL was a trypsin inhibitor in a previous paper was confirmed in this research. Many other proteins including lectins have been reported as being resistant to trypsin digestion (Lam *et al*., 2000; Gonzalez De Mejia and Prisecaru, 2005; Wong and Ng, 2005a). It would be interesting to determine if they are capable of inhibiting the ability of trypsin to digest other proteins or not in future.

All assays of porcine amylase activity, including inhibitory and stimulatory activities, were carried out by both methods (starch-iodine complex and

sugar assay) in this project. As some components in plant might have effect on starch-iodine complex formation, using both methods for amylase inhibitory activity determination seems necessary.

Interestingly, no PPA stimulatory activity was detected in extracts of the cotyledons at any stage of seed formation with the test tube assay suggesting that it might be masked by amylase inhibitor present in the same extracts. In extracts of immature whole seed, pods, seed coat and embryonic axis only PPA stimulatory activity was observed. Only in extracts of the embryonic axis amylase isoinhibitors bands were detectable on non-denaturing polyacrylmide gels. This suggests that detectable PPA stimulatory activity of embryonic axis extracts was dependent on the amount of amylase inhibitors in the extracts. It is also possible that other proteins (such as lectins other than PHA) would enhance activity of PPA. Therefore, the changes in PPA stimulators during seed formation need to be elucidated further using other methods like Western blotting. Gene expression of PHA in runner bean (*Phaseolus coccineus*) during seed development was found to be highly development-dependent and different levels were found in the cotyledons and embryonic axis (Voss *et al*., 1992).

No α-amylase isoinhibitor band was visible on non-denaturing polyacrylmide gels with extracts of the pod or seed coat. This is consistent with a previous report regarding absence of α-amylase inhibitor in extracts of the seed coat and pod using an immunoblotting method (Moreno *et al*., 1990). In a previous work, a sample of a dietary supplement which included common bean pod was found to have α-amylase inhibitory activity (Boniglia

et al., 2008). As the supplement contained other ingredients including other herb extracts or even α-amylase inhibitors added artificially to the supplement, it is not meaningful to compare results.

Chitinase activity was found in immature red kidney bean seeds after 10 days and increased in the cotyledons and embryonic axis during seed development with the same pattern. The chitinase activity decreased in the seed coat and pods at seed maturation. This coincides with development of the seed coat as a physical defence barrier to entry of pathogens or pests. Presence of chitinase in seed parts (including the seed coat, embryonic axis and cotyledons) of two common bean cultivars, soybean seed coat and Canadian cranberry bean whole seed has been demonstrated before (Ramos *et al*., 1998; Gijzen *et al*., 2001; Wang *et al*., 2009b). There was no prior report regarding chitinase activity in red kidney bean pods but it has been observed in pods of winged bean and pea before (Mauch *et al*., 1984; Sekeli *et al*., 2003).

Changes in β-1, 3- glucanase activity of red kidney bean seeds at the early stages of seed development were similar to those of chitinase activity. Their changes in the embryonic axis and cotyledons were also similar. Both activities decreased as the pod aged. In the seed coat the pattern of changes in β-1, 3- glucanase was different from that of chitinase. This is the first report regarding β-1, 3- glucanase activity in red kidney bean seed parts during seed formation.

Changes in AF activity in the cotyledons and embryonic axis were different from those of bioactive proteins during seed development of red kidney bean. This is in consistent with the data in chapter 3 indicating that the purified AFP does not have the selected biological activities. Therefore, the chitinase or β-1, 3- glucanase present in red kidney bean seeds do not contribute to AF activity against *A. alternata*. This is consistent with other reports. In a previous study, among different isoforms of chitinases and β-1, 3- glucanases isolated from tobacco leaves only some of them showed AF activity (Sela-Buurlage *et al*., 1993). No association between resistance of sorghum seed to grain mould and presence of chitinase and sormatin in the seed was found (Prom *et al*., 2005). In contrast, a β-1, 3- glucanase and a chitinase with antifungal activity against *Colletotrichum lindemuthianum* and *C. musae* were isolated from cowpea seeds (Gomes *et al*., 1996).

Chapter 7- Changes in Biological Activities During Germination of Red Kidney Bean Seeds in Response to UV Irradiation and Co-culturing with *Alternaria alternata*

7.1- Introduction

Plants are endowed with an arsenal of defence mechanisms to protect themselves against pathogens. These defence mechanisms can be induced in response to abiotic and biotic stressors (Muthukrishnan *et al*., 2001; Gomez *et al*., 2002; Sekeli *et al*., 2003). Chitinases and β-1, 3- glucanases, two examples of pathogenesis-related proteins (PR-proteins), are known to be induced by both kinds of stressors (Mohamed and Sehgal, 1997; Lima *et al*., 2002; Cota *et al*., 2007). These enzymes are found in vegetative tissues for protection against pathogen invasion (Cota *et al*., 2007) and in root exudates of cowpea (Nobrega *et al*., 2005). Chitinases can be involved in hydrolysis of fungal chitin, possibly leading to inhibition of fungal growth directly or indirectly by release of defence-related elicitors (Lima *et al*., 2002). A class IV chitinase was induced in tobacco plants when the plants were treated with an elicitor (β-1, 3-, 1,6-glucan) from *Alternaria alternata* (Shinya *et al*., 2007).

UV-C irradiation in previous investigations has been shown to induce all families of PR-Proteins (Green and Fluhr, 1995) including chitinase and β-1, 3- glucanase in peach and tomato fruits (Ghaouth *et al*., 2003; Charles *et al*.,

2009). Induction of these enzymes in tomato varieties by *A. alternata* has also been reported before (Cota *et al*., 2007). A chitinase with AF activity against *Rhizoctonia solani* was purified from ethylene-treated bean leaves (Benhamou *et al*., 1993). There is, however, little known about the effect of biotic and abiotic stress factors on bioactive proteins that occur in the different parts of a bean seed and seedling.

Plants also produce a variety of proteinaceous inhibitors of proteases and amylases that can be used against pathogens and predators (Mosolov *et al*., 2001a; Sanchez-Hernandez *et al*., 2004). Plant protease inhibitors have been considered as proteins that may function under biotic and abiotic stressful conditions (Pernas *et al*., 2000; Mosolov *et al*., 2001a and b). α-Amylase inhibitor biosynthesis was reported to be induced by abscisic acid (Domash *et al*., 2006), a stress hormone that can be produced by some fungi such as *A. alternata* (Crocoll *et al*., 1991). In response to abscisic acid as well as other biotic and abiotic stress factors trypsin and insect α-amylase inhibitors were induced in *Amaranthus hypochondriacus* leaves (Sanchez-Hernandez *et al*., 2004). Biotic and abiotic factors could affect AF activity in sunflower seeds during germination. A higher level of a lipid transfer protein with AF activity against *A. alternata* was found in sunflower seeds during salt stress, fungal infection or treatment with abscisic acid (Gonorazky *et al*., 2005).

Even though some papers have described changes in some antinutritional proteins during germination of bean seeds or changes in AFPs in response to stress in a specific tissue (Abeles *et al*., 1970; Sathe *et al*., 1983; Mauch *et al*., 1992; Savelkoul *et al*., 1994; Lange *et al*., 1996; Xue *et al*., 1998;

Kucera *et al*., 2003; Cuypers *et al*., 2005; Domash *et al*., 2006; Sangronis and Machado, 2007; Campos *et al*., 2009), there is a lack of comprehensive understanding about changes in AF activity in different parts of seeds and seedlings as well as root exudates during germination or in response to biotic and abiotic stress factors. There is no report about presence of bioactive proteins in seed and root exudates of red kidney bean although other leguminous seeds have been reported to release trypsin/chemotrypsin inhibitors during imbibition (Mosolov *et al*., 2001a). In this project, red kidney bean seedlings developed under two different sets of stress treatments: UV irradiation daily or co-culture with *Alternaria alternata*. The following changes in red kidney bean seed/seedling parts and exudates of seeds and roots under these conditions were studied: AF activity against *A. alternata* as well as specific activities of chitinase, β-1, 3- glucanase, bovine trypsin inhibitor and porcine α-amylase inhibitor.

7.2- Materials and methods

7.2.1- Ultraviolet (UV) irradiation of red kidney bean seeds germinated under aseptic conditions

Red kidney bean seeds were surface-disinfected by soaking in 10% (v/v) commercial bleach and rinsed three times with sterile distilled water before they were placed in sterile tissue culture jars (0.25 litre capacity) containing 60 ml of 1.6% (w/v) agar. After incubation for 16 h in the dark at 26 ± 1^{0}C, the seeds or seedlings kept in culture jars were exposed twice daily, 30 min each time of UV radiation (UV-C with an intensity of 0.0725 w/m^2 and 253.7 nm wavelength from a bulb of TUV 30 W, G30TB supplied by Philips, The Netherlands) inside a laminar flow cabinet. During irradiation,

the lids of the culture jars were taken off. There was a 30 min pause between the two irradiation treatments and during this break the jars were returned to the 26^0C room (Lima *et al*., 2002). For control, seeds and seedlings were protected by aluminium foil from irradiation (Green and Fluhr, 1995). After the second UV irradiation treatment on an appropriate sample collection day, seeds and seedlings were incubated at the 26^0C dark room for four more hours before proteins were extracted (Fig. 7.1). Proteins were extracted as described in 2.2 from the seed and seedling parts including the hypocotyl, leaves, cotyledons and root at 0, 1, 2, 5, 7 and 11 days after sowing seeds. The agar was also extracted to yield seed or root exudates (Rose *et al*., 2006).

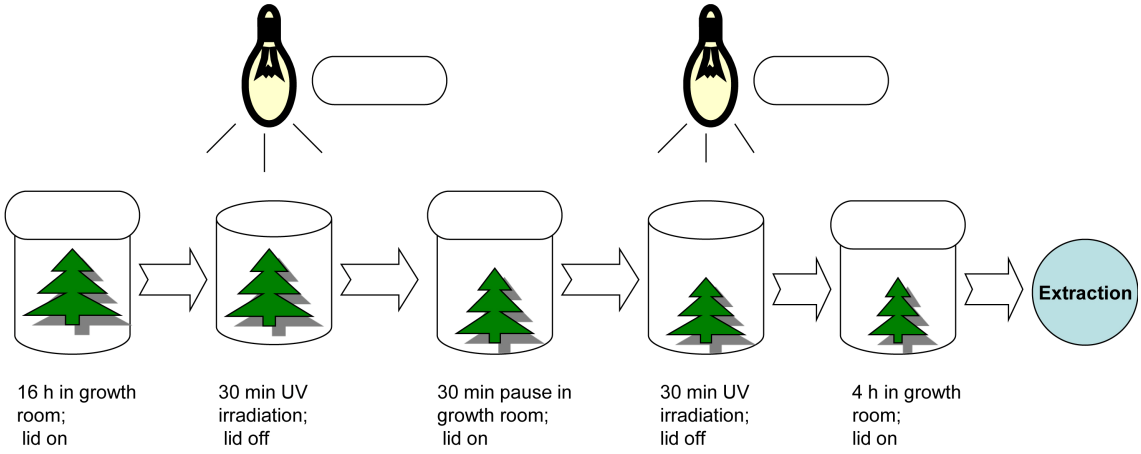

Figure 7.1- UV irradiation scheme for day zero. After this day, the seed and seedling parts were treated daily and harvested and extracted on days 1, 2, 5, 7 and 11. A broadband UV radiometer (UV-Biometer, model 501A, Solar Light Company, PA, USA) was used to measure UV-C radiation.

7.2.2- Development of red kidney bean seedlings in the presence of *A. alternata* (co-culture experiments)

Alternaria alternata was cultured in sterile tissue culture jars (0.25 litre capacity) containing 60 ml potato dextrose agar. Red kidney bean seeds were prepared as described 7.2.1 and germinated in sterile tissue culture jars containing 0.89% agar (w/v) for two days at 26 ± 1^{0}C in the dark. After germination, they were then transferred to culture jars containing PDA (potato dextrose agar) and placed equidistant (2 cm) from the growing margin of the fungal colony. For control, the seeds were placed in jars containing PDA without the fungal culture (Gonorazky *et al*., 2005). All jars were returned to the same growth room and after 4 h the day zero samples were extracted. Seed and seedling parts including hypocotyl, leaves, cotyledons and roots were harvested at 1, 2, 5, 7 and 11 days after incubation and extracted as described in 2.2. The PDA was extracted to yield seed and root exudates (Rose *et al*., 2006).

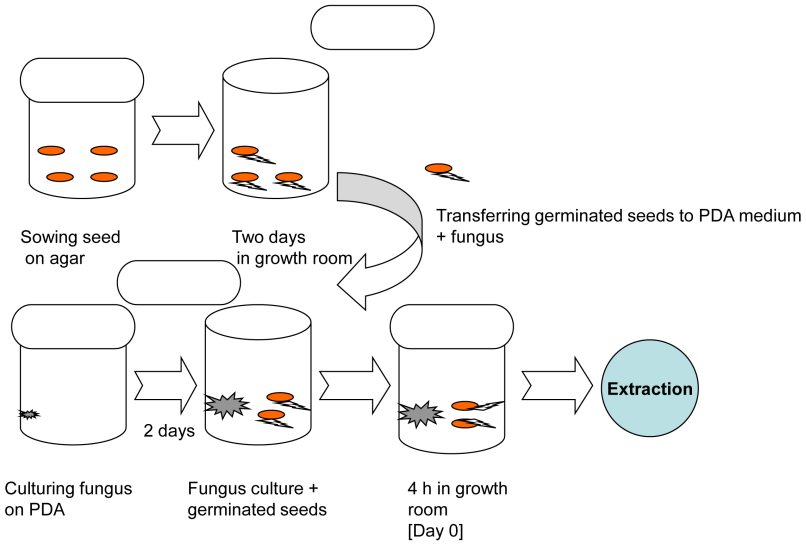

Figure 7.2- Preparation and co-culture of seeds with *Alternaria alternata*. Proteins were extracted from seed or seedling parts at day 0 or after incubation in a growth room for 1, 2, 5, 7 and 11 days.

7.2.3- Assays of antifungal and biochemical activities

All seed and seedling parts were separated, extracted as described in 2.2 and their antifungal and biochemical activities were assessed as described in 2.6 and 2.7. Protein content was measured as described in 2.4.

7.3- Results

7.3.1- Effect of biotic and abiotic stress factors

7.3.1.1- Effects of UV irradiation

7.3.1.1.1- Seedling growth and changes in AF (antifungal) activity

The length of red kidney bean seedlings was affected by UV irradiation in comparison with control (Fig. 7.3). AF activity in extracts of the cotyledons treated with UV irradiation did not differ significantly from control and it did not change in the first 7 days after start of imbibition but was lower after 11 days (Figs. 7.4A and 7.5). In the embryonic axis, it did not change during

germination or in response to UV irradiation (Fig. 7.4B and 7.6). In the dry seed coat, there was no AF activity (data not shown) but it was detected during imbibition and increased within the first day of germination before it decreased in the following day. No difference was observed between the UV treatment and control (Fig. 7.4C). There was AF activity in seed and root exudates during germination which peaked at day 2 and then remained at this level. No difference in AF activity was observed between plants treated with UV irradiation and control (Fig. 7.4D). A relatively low level of AF activity was detected in root extracts during germination. It increased after 5 days in both the UV treatment and control but there was no difference between the two treatments (Fig. 7.4E). In hypocotyl extracts a very low level of AF activity was also detected except at day 7 when it was increased in response to UV irradiation (Figs. 7.4F).

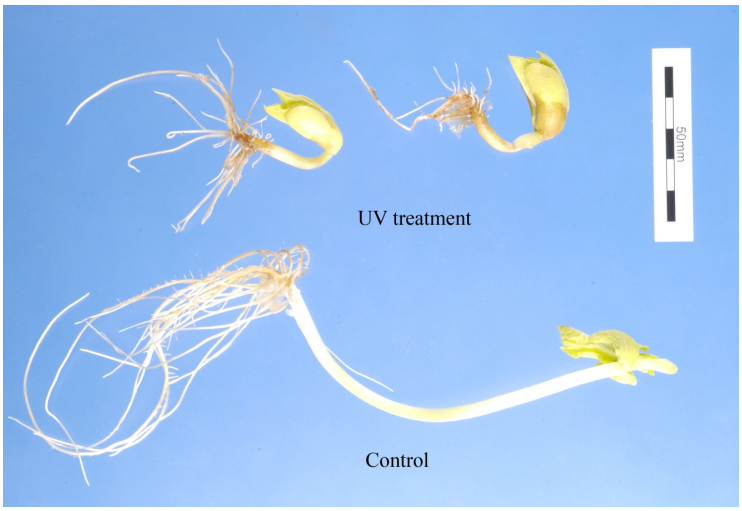

Figure 7.3- Effect of UV on seedling length. Seedlings were harvested after 7 days from irradiation with UV as described in the methods.

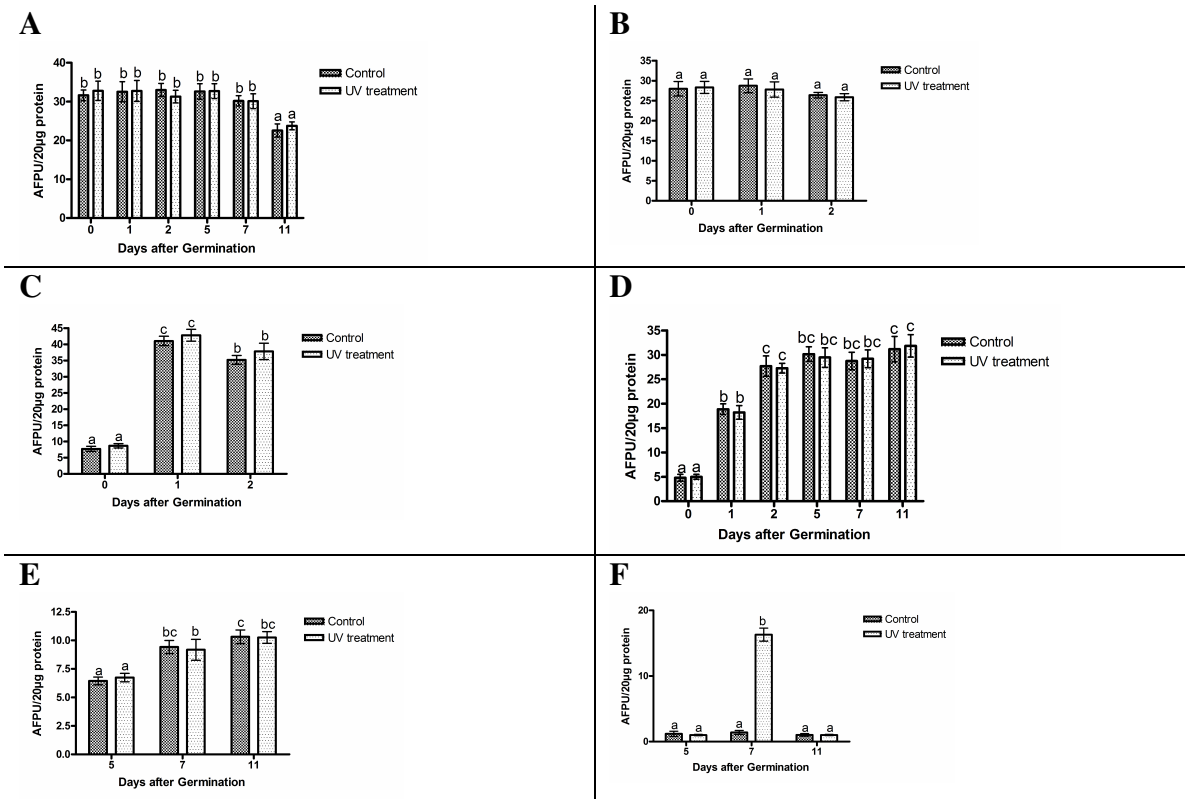

Figure 7.4- Changes in antifungal activity in extracts of red kidney bean **A-** Cotyledons, **B-** Embryonic axis, **C-** Seed coat, **D-** Seed and root exudates, **E-** Root and **F-** Hypocotyl in response to UV treatment during seed germination. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

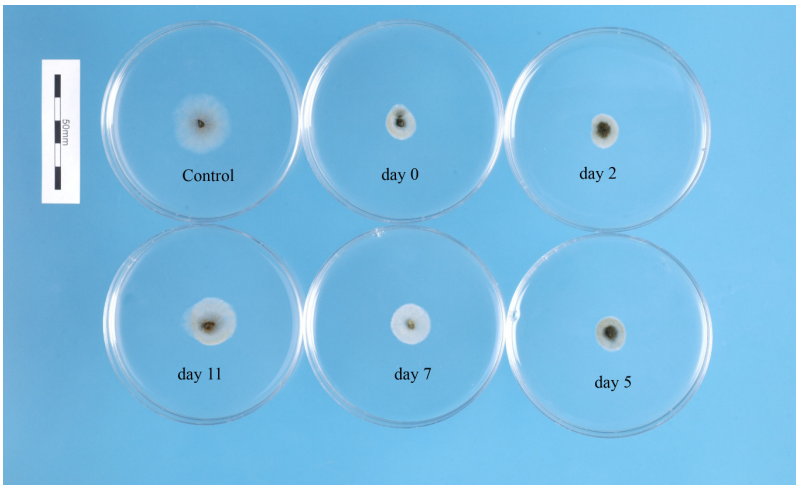

Figure 7.5- Changes in the area of *A. alternata* colonies in the presence of antifungal protein of red kidney bean cotyledons during seed germination.

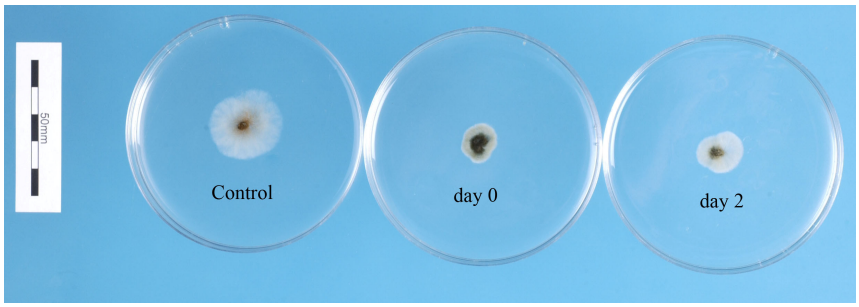

Figure 7.6- Changes in the area of *A. alternata* colonies in the presence of antifungal protein of red kidney bean embryonic axis during seed germination.

AF activity was not detectable in leaf extracts of the control or UV irradiation treatments in the first 7 days. Those of the UV-treated seedlings harvested at day 11 had higher AF activity than those of the control (data not shown).

7.3.1.1.2- Changes in bovine trypsin inhibitory activity

In the control, trypsin inhibitory activity in crude cotyledonary extracts first increased, then decreased after 5 days and remained at this low level until 11 days. In the UV treatment, there was a higher level of the trypsin inhibitory activity than the control after day 5 (Fig. 7.7A). The level of trypsin inhibitory activity in the embryonic axis increased during germination. UV irradiation had no effect on this (Fig. 7.7B). In the seed coat there was a low level of trypsin inhibitory activity which did not change after 1 day of germination. UV irradiation also had no effect on this (Fig. 7.7C). In seed and root exudates, trypsin inhibitory activity decreased slightly during the first two days of germination before it began to increase. There was a higher level of the inhibitory activity in the UV treatment compared to control after day 5 (Fig. 7.7D).

In the root, trypsin inhibitory activity decreased after day 7. UV irradiation increased this significantly in comparison with control (Fig. 7.7E). Extracts from the hypocotyl showed trypsin inhibitory activity which decreased during germination. There was a higher level of the inhibitory activity in the UV treatment compared to control (Fig. 7.7F). Trypsin inhibitory activity in leaf extracts decreased during germination. UV irradiation increased the activity after 5 days of germination (Fig. 7.7G).

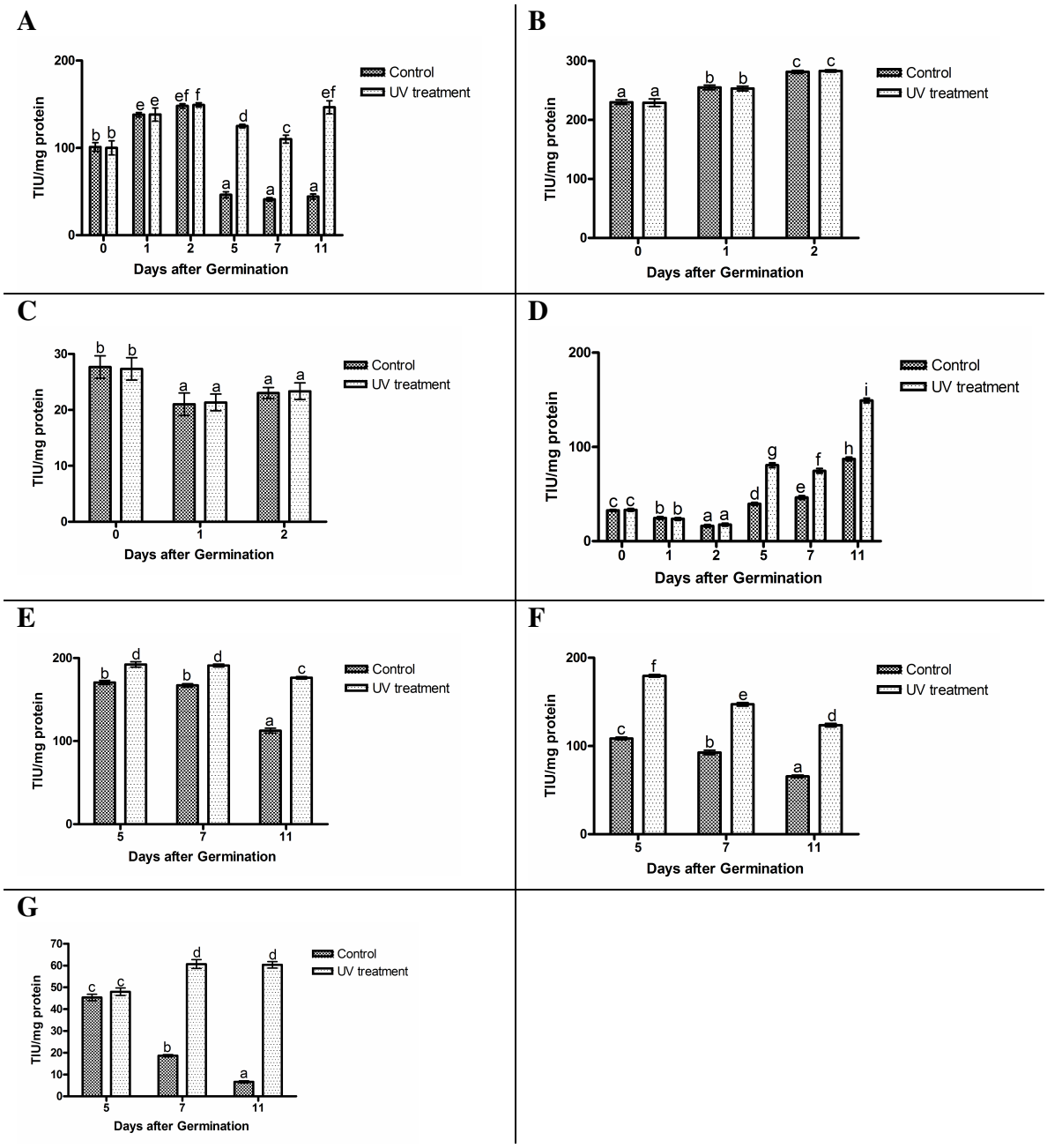

Figure 7.7- Changes in bovine trypsin inhibitory activity in extracts of red kidney bean **A-** Cotyledons, **B-** Embryonic axis, **C-** Seed coat, **D-** Seed and root exudates, **E-** Root, **F-** Hypocotyls and **G-** Leaves in response to UV treatment during seed germination. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

7.3.1.1.3- Effect of UV irradiation on changes in porcine α-amylase inhibitory/stimulatory activity

Compared to control, the cotyledon in the UV irradiation treatment had less inhibitory activity in the first 2 days (Fig. 7.8). From day 5, the cotyledonary extracts of both control and the UV treatment exhibited the same levels of amylase stimulatory activity (Fig. 7.8). Nevertheless, in-gel detection demonstrated the presence of amylase isoinhibitors in the cotyledonary extracts (Fig. 7.9).

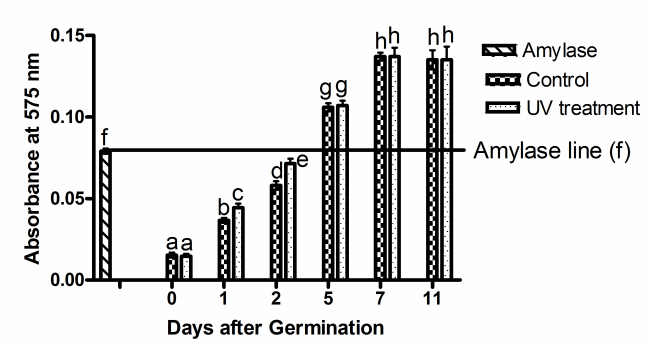

Figure 7.8- Changes in α-amylase inhibitory/stimulatory activity in extracts of red kidney bean cotyledons in response to UV treatment during seed germination. A higher absorbance in the graph shows more sugar release or more amylolytic activity. Amylase line was PPA activity in the absence of any extract. Activities above or below the line showing PPA activity (amylase line) were PPA stimulatory or inhibitory activities, respectively. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

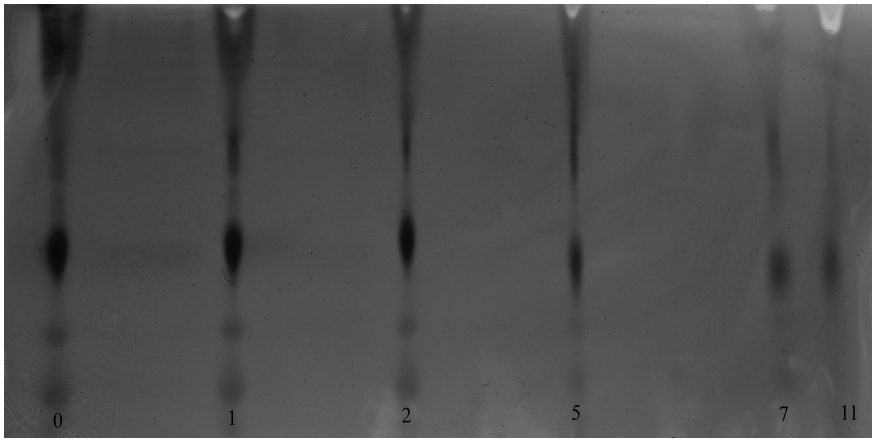

Figure 7.9- Detection of isoinhibitors of PPA activity following non-denaturing PAGE of extracts from red kidney bean cotyledon during seed germination (0, 1, 2, 5, 7 and 11 days). Equal amount of protein (30 μg) was loaded in each lane.

Presence of amylase isoinhibitors in extracts of the UV-irradiated embryonic axis was found using the in-gel electrophoretic detection method (data not shown). However, no amylase inhibitory activity was detectable in extracts of the embryonic axis using either the reducing sugar or starch-iodine complex assays. Instead, amylase stimulatory activity was detected and increased at day 2 in the embryonic axis of the UV treatment and control (Fig. 7.10A). Amylase stimulatory activity in the seed coat decreased following start of imbibition. No difference in this activity was observed between UV irradiation treatment and control (Fig. 7.10B). No amylase isoinhibitor was found in seed coat extracts by the in-gel electrophoretic detection method (data not shown). No amylase inhibitory activity was found in seed or root exudates by either the test tube assay or the in-gel electrophoretic method (data not shown). During germination, amylase stimulatory activity increased in the exudates and peaked at day 7. There was a lower level of this activity in the UV irradiation compared with control (Fig. 7.10C). No amylase inhibitory activity was detected in root extracts using the in-gel electrophoretic detection method (data not shown).

Amylase stimulatory activity increased after germination but there were lower levels of the activity in response to the UV irradiation treatment compared with control (Fig. 7.10D). In the hypocotyl, amylase stimulatory activity increased at day 7 and then decreased at day 11. The levels of this activity in the UV irradiation treatment were only significantly lower than those of control after 5 and 7 days of germination (Fig. 7.10E). No amylase isoinhibitor was detected in hypocotyl extracts using the in-gel electrophoretic detection method (data not shown). No amylase inhibitory activity was found in leaf extracts (in-gel electrophoresis data not shown). In both control and the UV irradiation treatment, there was no difference in the levels of amylase stimulatory activity in leaf extracts and this activity decreased during germination (Fig. 7.10F).

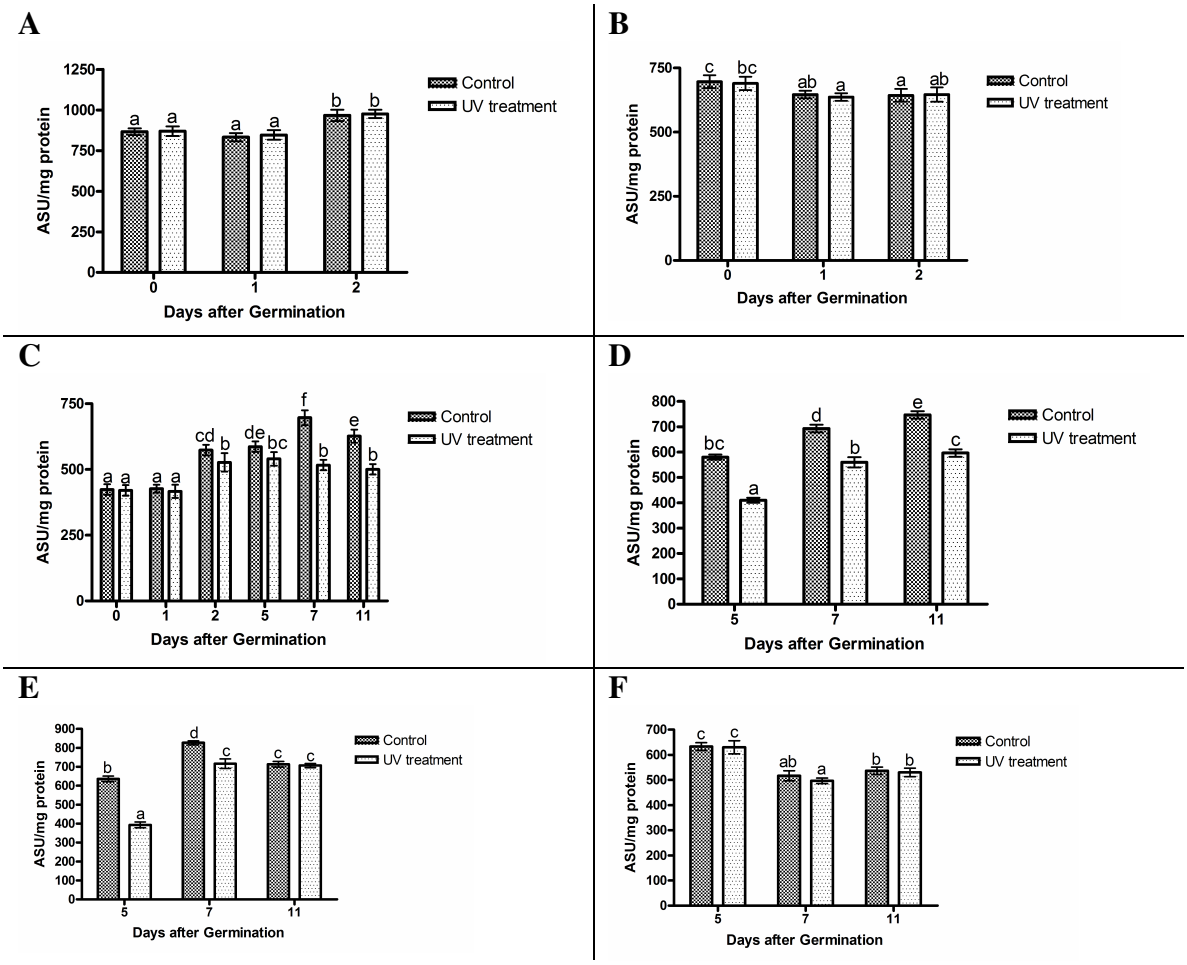

Figure 7.10- Changes in α-amylase stimulatory activity in extracts of red kidney bean **A-** Embryonic axis, **B-** Seed coat, **C-** Seed and root exudates, **D-** Root, **E-** Hypocotyls and **F-** Leaves in response to UV treatment during seed germination. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

7.3.1.1.4- Changes in chitinase activity

Chitinase activity in the cotyledons decreased after 1 day of germination, then it increased until day 5 before it decreased slightly. The enzyme activity was enhanced in the cotyledons exposed to UV irradiation in comparison to control (Fig. 7.11A). Chitinase activity increased in the embryonic axis and seed coat during seed germination. Higher levels of the enzyme activity were found in extracts from these seed parts in response to the UV

irradiation treatment (Figs. 7.11B and C). Seed and root exudates had detectable chitinase activity during germination. The enzyme activity increased in the exudates after one day of germination and decreased at day 2 before it increased significantly again. In the UV irradiation treatment there were higher levels of the enzyme activity in comparison with control (Fig. 7.11D). Chitinase activity increased in the root during germination. In response to UV irradiation there were higher levels of the enzyme activity than control (Fig. 7.11E). Chitinase activity decreased in the hypocotyl and leaves at day 7 before it increased at day 11 during germination. While in the UV irradiation treatment of the hypocotyl, the enzyme activity only increased at day 11 (Figs. 7.11F and G). In response to UV irradiation, the enzyme activity was enhanced in the hypocotyl and leaves compared to control during germination.

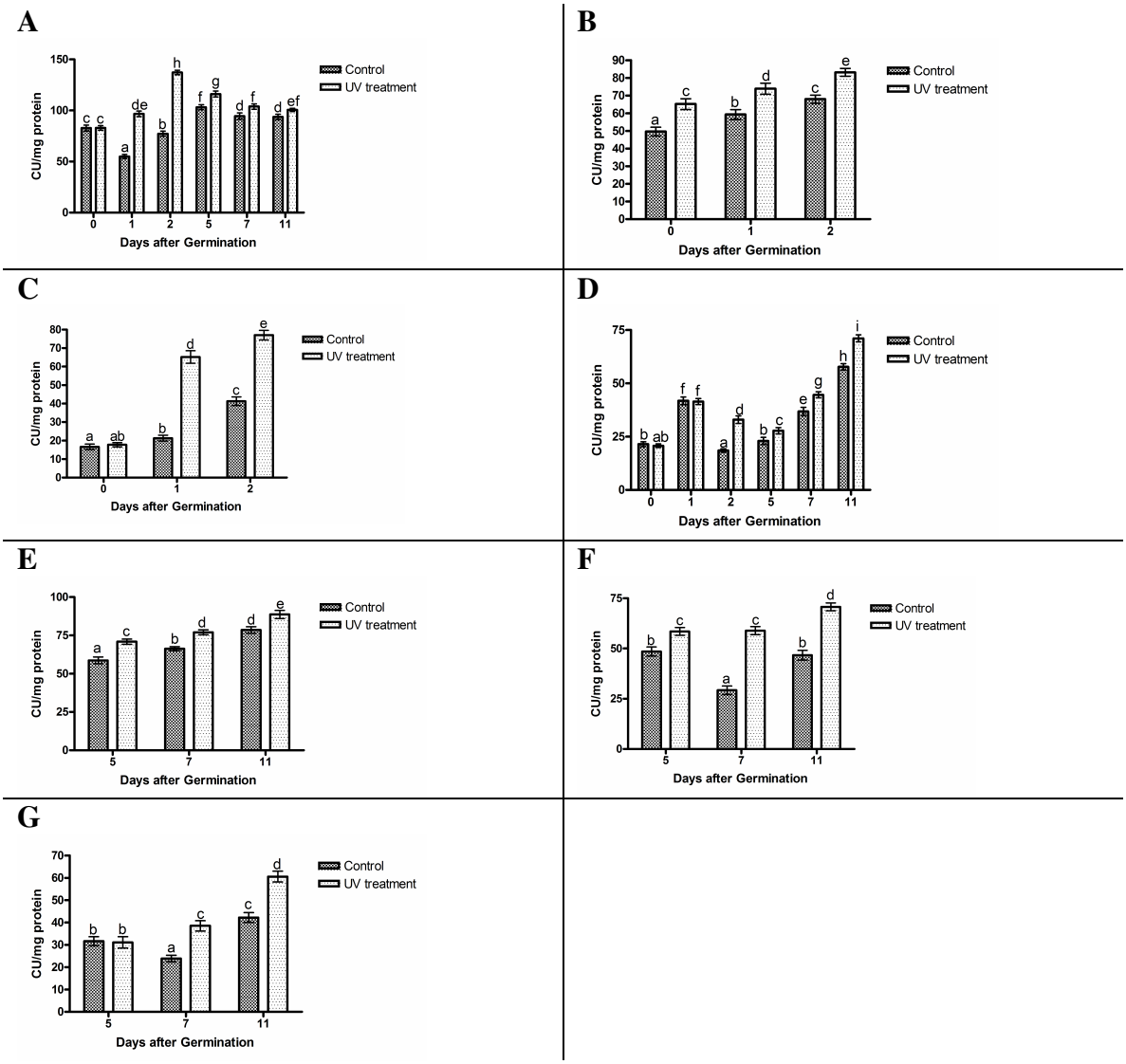

Figure 7.11- Changes in chitinase activity in extracts of red kidney bean **A-** Cotyledons, **B-** Embryonic axis, **C-** Seed coat, **D-** Seed and root exudates **E-** Root, **F-** Hypocotyls and **G-** Leaves in response to UV treatment during seed germination. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

7.3.1.1.5- Changes in β-1, 3- glucanase activity

There was no difference in the levels of glucanase activity in the cotyledons after 1 day of germination. Then there were higher levels of the enzyme activity in response to UV irradiation than control. In control, the enzyme activity increased at day 1 and then declined gradually until day 7 before it

increased again at day 11. In the UV irradiation treatment, the changes in the enzyme activity during germination were similar except that the levels of the enzyme activity were the same at day 2 and 5 (Fig. 7.12A). Glucanase activity in the embryonic axis decreased during germination. There was no difference between the UV irradiation treatment and control (data not shown). In the seed coat, there was a low level of the enzyme activity at day zero which was then no longer detectable after germination. The UV irradiation treatment did not have an effect on the enzyme activity in the seed coat (data not shown). In seed and root exudates the enzyme activity was higher at day 5 than at day 7 and it then increased until day 11. There was no significant difference between UV treatment and control (data not shown). In the root the enzyme activity increased slightly during germination with or without the UV irradiation treatment. At day 11, a promotive effect of the UV irradiation treatment on the enzyme activity in the root was found (Fig. 7.12B). In the hypocotyl and leaves the enzyme activity was highest at day 11. There was no difference between UV treatment and control (data not shown).

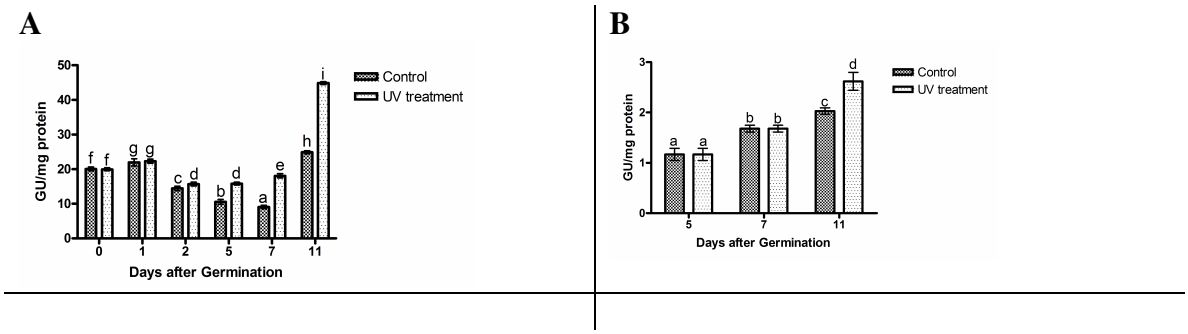

Figure 7.12- Changes in β-1, 3- glucanase activity in extracts of red kidney bean **A-** Cotyledons and **B-** Root in response to UV treatment during seed germination. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

7.3.1.2- Effects of co-culturing red kidney bean seedlings with *A. alternata*

7.3.1.2.1- Seedling growth and changes in antifungal activity

Growth of the seedlings was reduced in response to co-culturing with *A. alternata* when compared to control (data not shown). The levels of antifungal activity in extracts of the cotyledons from seedlings co-cultured with *A. alternata* and control were the same and did not change in the first 7 days of seed germination. At day 11, there was a similar significant drop in the AF activity of the cotyledons regardless whether the seedlings were co-cultured with the fungus or not (Fig. 7.13A). The AF activity in the embryonic axis and seed coat did not change during co-culturing of the germinated seeds in the presence or absence of *A. alternata* (Figs. 7.13B and C). Interestingly, AF activity in seed and root exudates during co-culturing of the seedlings with the fungus was increased after 2 days of germination compared to control (Fig. 7.13D). The root had a low level of AF activity which was higher in that of the seedlings co-cultured with the fungus than in control (Fig. 7.13E).

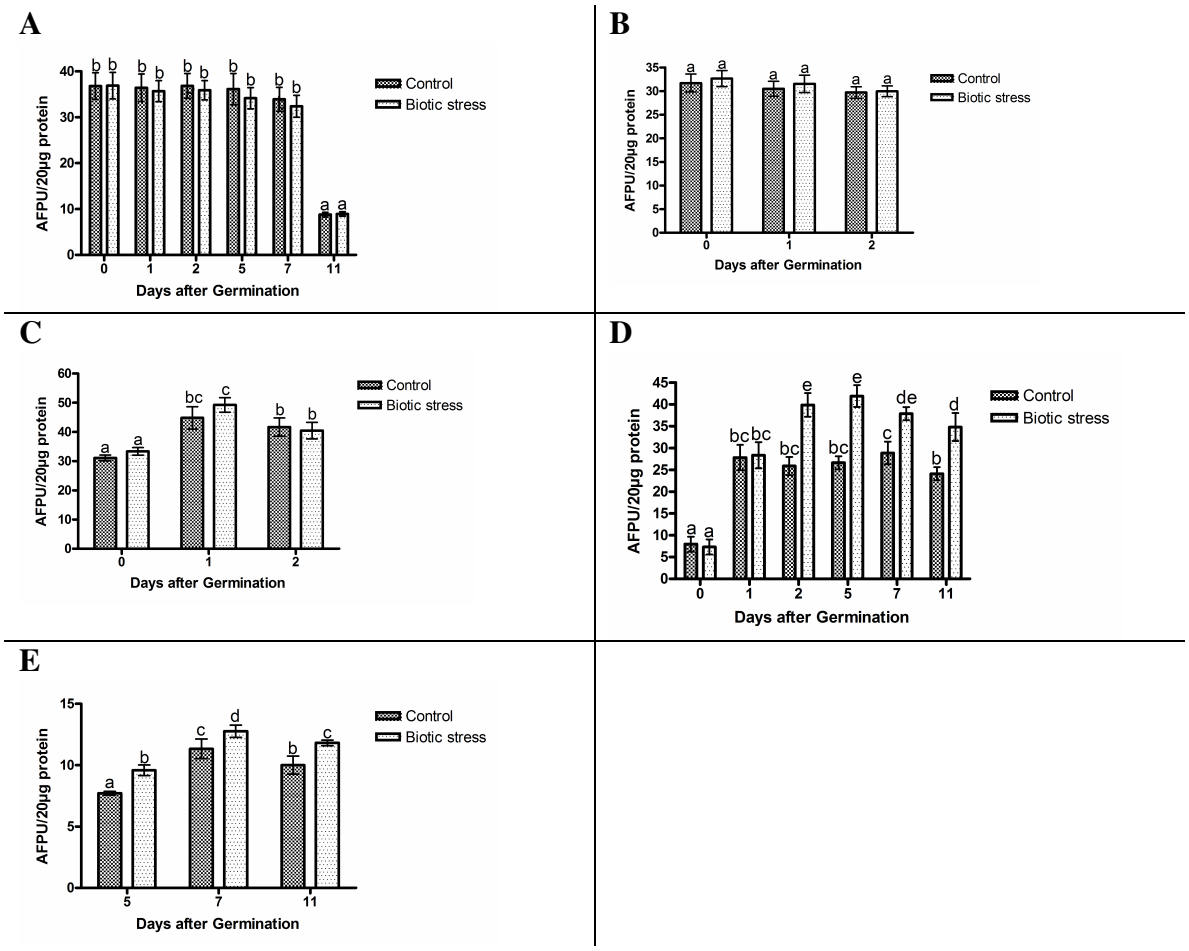

Figure 7.13- Changes in antifungal activity in extracts of red kidney bean **A-** Cotyledons **B-** Embryonic axis, **C-** Seed coat, **D-** Seed and root exudates and **E-** Root in response to co-culturing of red kidney bean seedlings with or without *A. alternata*. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

The hypocotyl had a low level of AF activity which was the same whether the seedlings were co-cultured with the fungus or not (data not shown). AF activity was not detectable in the leaves until day 11. There was no significant difference in the levels of AF activity in the leaves between co-culture treatment with the fungus and control (data not shown).

7.3.1.2.2- Changes in trypsin inhibitory activity

Co-culture with the fungus had no effect on the level of trypsin inhibitory (TI) activity in the cotyledons and embryonic axis during seed germination (Figs. 7.14A and B). TI activity was higher in the seed coat, seed and root exudates, the root, the hypocotyl and leaves during germination of red kidney bean in response to co-culturing with the fungus (Figs. 7.14C-G).

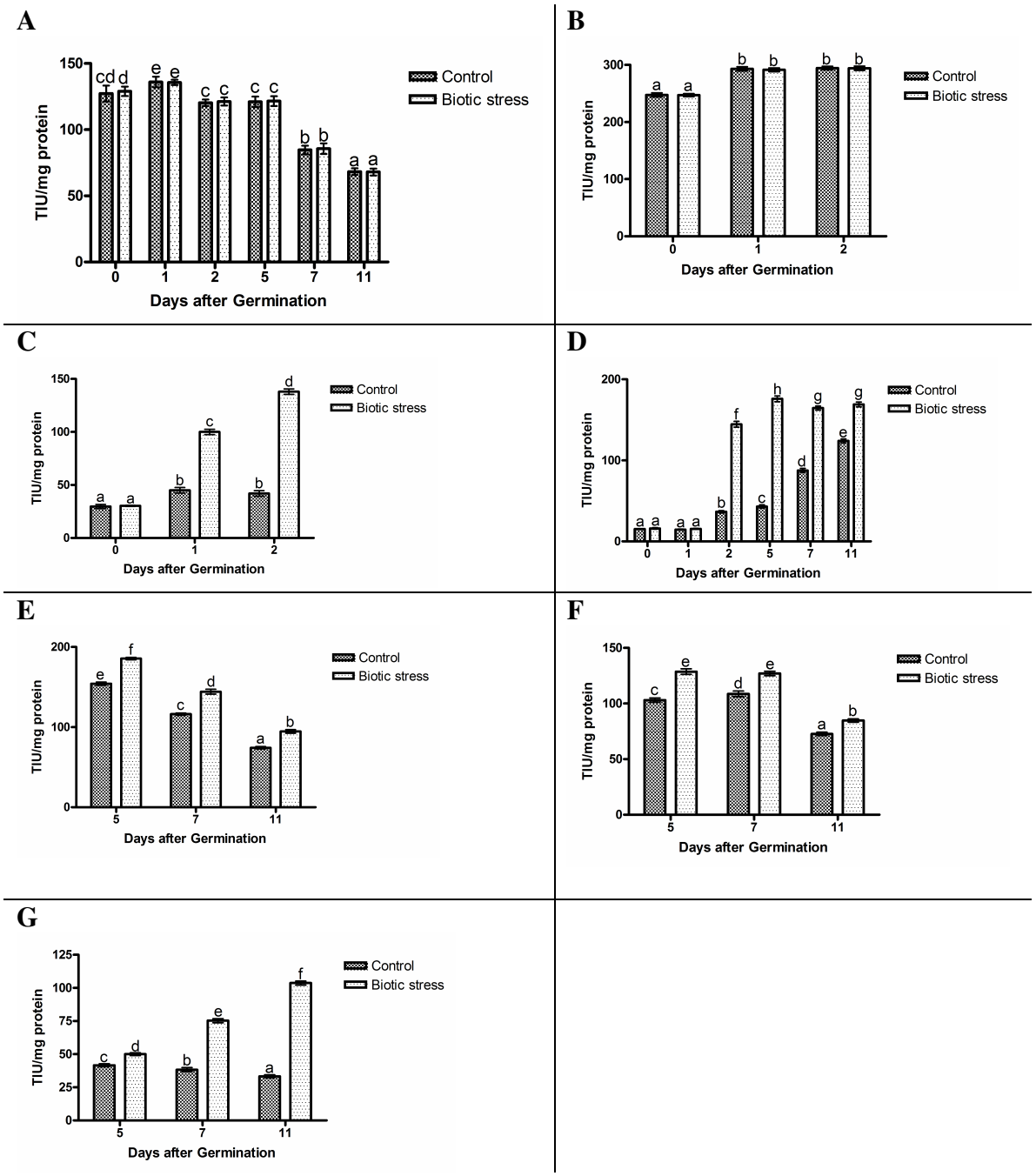

Figure 7.14- Changes in bovine trypsin inhibitory activity in extracts of red kidney bean **A-** Cotyledons **B-** Embryonic axis, **C-** Seed coat, **D-** Seed and root exudates, **E-** Root, **F-** Hypocotyls and **G-** Leaves in response to co-culturing of red kidney bean seedlings with or without *A. alternata*. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

7.3.1.2.3- Changes in α-amylase stimulatory/inhibitory activity

Amylase inhibitory (AI) activity was found in extracts of the cotyledons at day zero but during germination it was masked by amylase stimulatory activity (Fig. 7.15). There was no difference between the co-culture treatment with the fungus and control. Gel electrophoresis of cotyledonary extracts confirmed the presence of AI activity during germination up to day 11 (data not shown). Co-culturing the seedlings with the fungus had no effect on the level of AI activity in the cotyledons during seed germination.

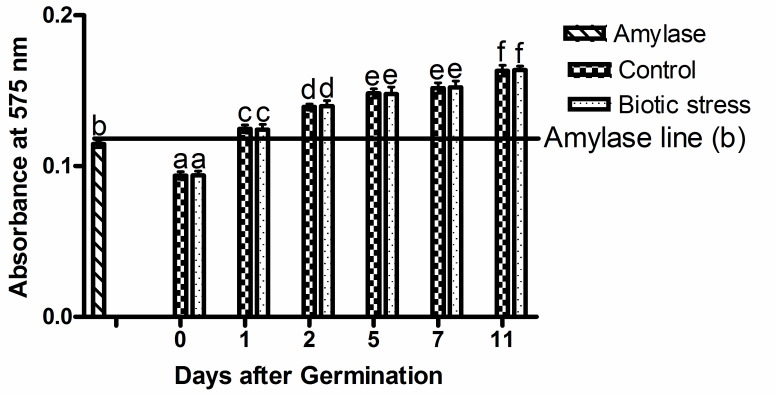

Figure 7.15- Changes in α-amylase inhibitory/stimulatory activity in cotyledons of red kidney bean in response to co-culturing of red kidney bean seedlings with or without *A. alternata*. A higher absorbance in the graph shows more sugar release or more amylolytic activity. Amylase line was PPA activity in the absence of any extract. Activities above or below the line showing PPA activity (amylase line) were amylase stimulatory or inhibitory activity, respectively. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

There was a slightly lower level of amylase stimulatory activity in the embryonic axis of the germinated seeds co-cultured with the fungus during seed germination (Fig. 7.16A). Co-culturing the seedlings with the fungus had no effect on the level of amylase stimulatory (AS) activity in the seed coat (Fig. 7.16B). In the seedlings co-cultured with the fungus, there were lower levels of AS activity in seed or root exudates (Fig. 7.16C), the root

(Fig. 7.16D) and hypocotyl (Fig. 7.16E). There was no significant difference in the level of AS activity between the leaves of the seedlings co-cultured with the fungus and those of control (data not shown).

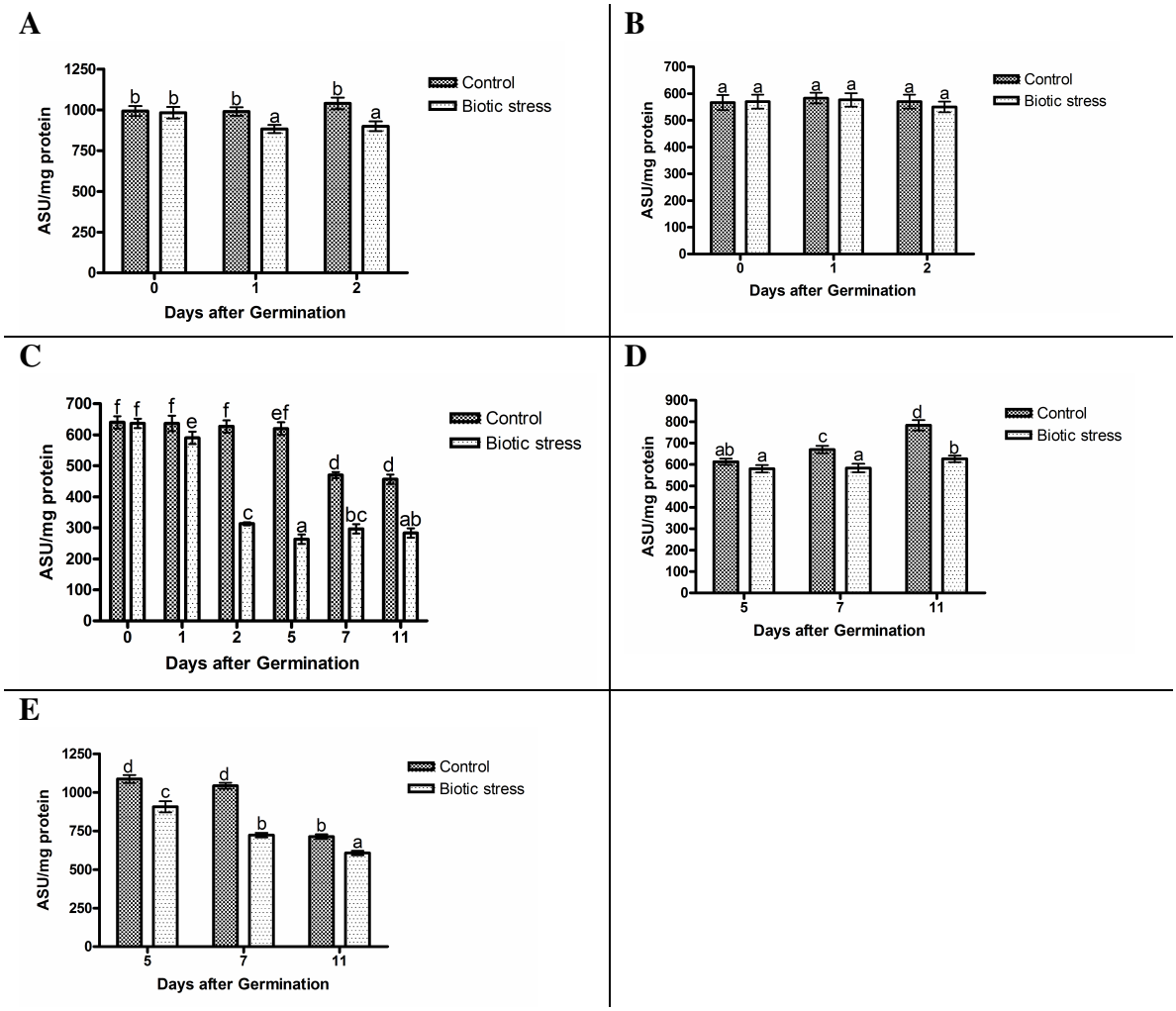

Figure 7.16- Changes in α-amylase stimulatory activity in extracts of red kidney bean **A-** Embryonic axis, **B-** Seed coat, **C-** Seed and root exudates, **D-** Root and **E-** Hypocotyl in response to co-culturing of red kidney bean seedlings with or without *A. alternata*. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

7.3.1.2.4- Changes in chitinase activity

Co-culturing of the seedlings with the fungus had no effect on the levels of chitinase activity in the cotyledons and embryonic axis (Figs. 7.17A and B). In the treatment of co-culturing with the fungus, there were higher levels of chitinase activity in the seed coat (Fig. 7.17C), seed and root exudates (Fig. 7.17D), root (Fig. 7.17E) and leaves (Fig. 7.17F). This effect was not observed in the hypocotyl (data not shown).

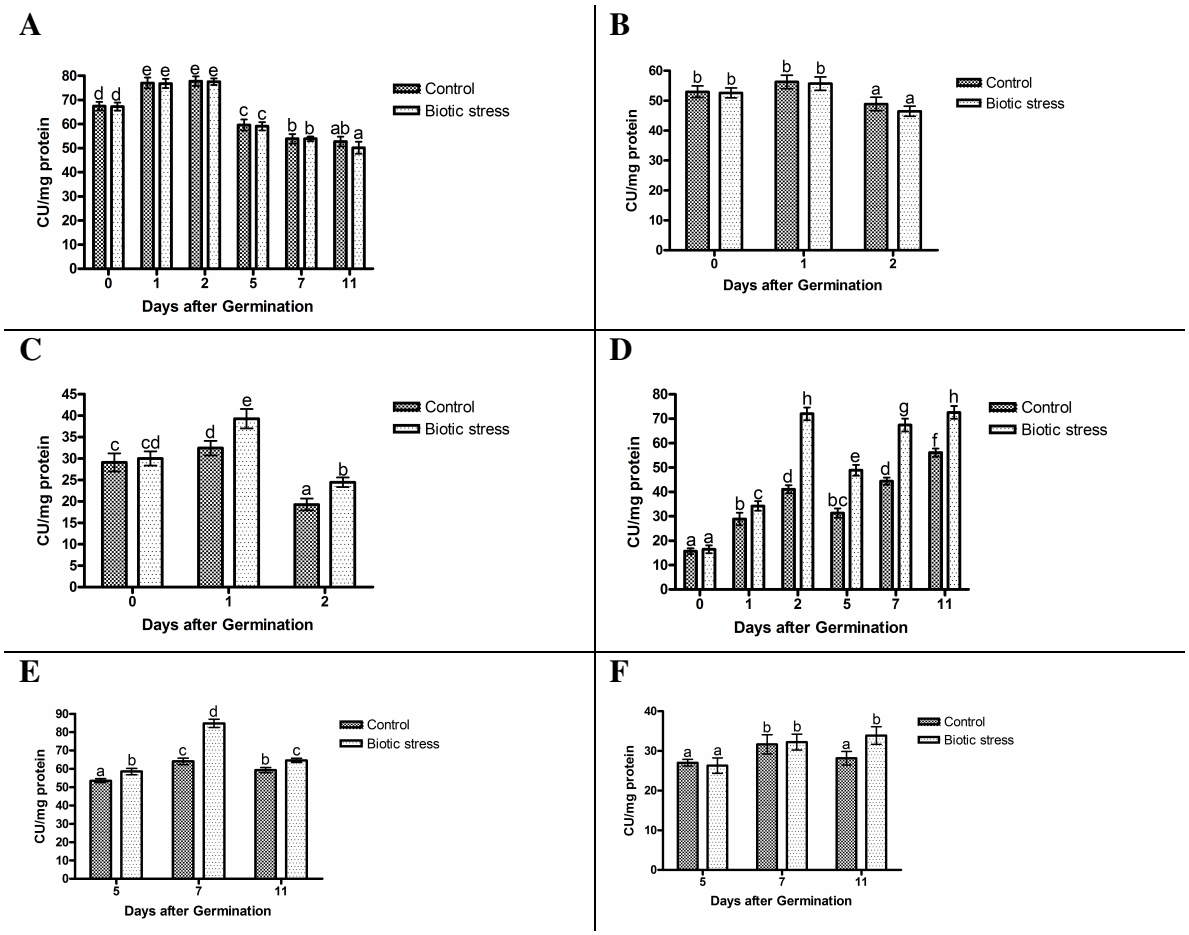

Figure 7.17- Changes in chitinase activity in extracts of red kidney bean **A-** Cotyledons, **B-** Embryonic axis, **C-** Seed coat, **D-** Seed and root exudates, **E-** Root and **F-** Leaves in response to co-culturing of red kidney bean seedlings with or without *A. alternata*. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

7.3.1.2.5- Changes in β-1, 3- glucanase activity

Co-culturing the seedlings with the fungus had no effect on the levels of β-1, 3- glucanase activity in the cotyledons, the embryonic axis, seed and root exudates, the root and hypocotyl (data not shown). Interestingly, the enzyme activity was enhanced in the leaves in the co-culture treatment (Fig. 7.18).

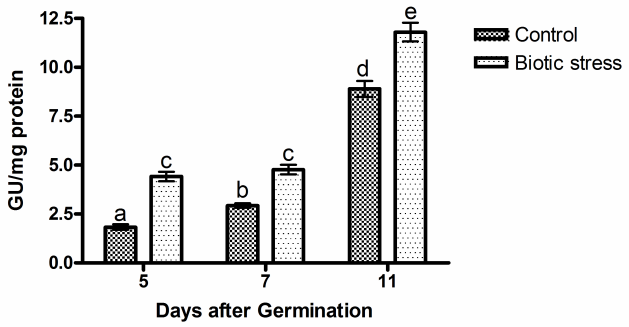

Figure 7.18- Changes in specific β-1, 3- glucanase activity in leaves of red kidney bean in response to co-culturing of red kidney bean seedlings with or without *A. alternata*. Means labeled with the same letter do not differ significantly according to Duncan's MRT ($P<0.05$).

7.4- Discussion

The abiotic (UV-C irradiation) and biotic (co-culturing with *Alternaria alternata*) treatments retarded growth of red kidney bean seedlings. It has been shown that UV irradiation stress affected plant growth and development adversely (Kuhlmann and Muller, 2009).

In preliminary experiments, the following problem was encountered. When the seeds were placed directly on the media already cultured with *A. alternata*, they would not germinate. This might be related to the ability of the fungus to produce abscisic acid (Crocoll *et al.*, 1991) which is a well-

known inhibitor of seed germination. To solve this problem, seeds were germinated first for two days before they were transferred to PDA jars containing the growing fungus. Although the effect of ABA produced by the fungus during co-culture was not the primary study objective here, it seems worthwhile to investigate if ABA might influence the bioactivities in the extracts of red kidney bean seedlings in future studies.

There was no AF activity in the dry seed coat of red kidney bean but after imbibition it became detectable. This might be attributed to exudates from other seed parts. Presence of AF activity in red kidney bean seed and root exudates was shown here for the first time. It is also known that cowpea root exudates have AF activity (Nobrega *et al*., 2005). Occurrence of antifungal proteins has been reported before in the seed coat of some seeds such as soybean (Santos *et al*., 2008). Induction of an AFP in corn kernels during imbibition and early stage of germination has also been demonstrated before (Guo *et al*., 1997).

It has been shown that some endogenous proteases can act on some seed proteins to produce new peptide fragments with AF activity. The AFPs generated in this way along with pre-existing AFPs play an important role in plant defence during seed germination (Wang *et al*., 2002). More experiments can be done regarding isolation and identification of AFPs in the seed coat and exudates of red kidney bean after imbibition to see whether they are the same as those present in the cotyledons and embryonic axis or not. AF activity in the roots of red kidney bean seedlings was very low suggesting that the AFP there might have been leached out.

UV irradiation increased the AF activity in the leaves of red kidney bean seedlings at day 11 but it is not known whether this activity was contributed by a single AFP or different peptide fragments capable of growth inhibition of *A. alternata*. The AF activity in the hypocotyl was increased by the irradiation treatment at day 7 but not at day 11. This might be attributed to damage of the hypocotyl at day 11 by the UV irradiation treatment. Apart from the leaves and hypocotyl, the UV irradiation treatment had no effect on AF activity in other parts of red kidney bean seedlings, suggesting no induction of AFP formation in these seedling parts.

Some antifungal proteins might have been induced in red kidney bean seedlings in response to the UV irradiation treatment. A question arises as to whether the seedlings could become more resistant to some pathogens after a short period of UV irradiation or not. It might be hypothesized that if a stress like UV irradiation is applied to seedlings in a glasshouse, a high rate of disease resistance can be induced in the seedlings against some pathogens before transplanting. As induced resistance is non-specific (Agrios, 1988), it might act against different pathogens. UV-B irradiation has reduced severity of drought stress in pea plants in a previous study (Nogues *et al*., 1998) although it has also been reported to diminish flower production in some plants (Sampson and Cane, 1999). Similar research has been successful in making the fruit of tomatoes resistant against some postharvest fungal pathogens including *Alternaria alternata*, *Botrytis cinerea* and *Rhizopus stolonifer* (Charles *et al*., 2009). In other research, shelf-life of a variety of lettuce has been extended by UV-C irradiation. It has been attributed to

microflora inhibition by the treatment (Allende *et al*., 2006) even though antifungal protein changes have not been assessed. UV irradiation must be at a level that does not cause any damage to plant tissue or yield reduction. UV-B would be appropriate although stronger induction of disease resistance has been reported by UV-C (Kucera *et al*., 2003). It would also be of interest to determine how long PR-proteins could persist in a plant tissue.

Co-culturing of germinated red kidney bean seeds with *A. alternata* only increased AF activity in seed and root exudates as well as extracts of the root but not those of other parts of red kidney bean seedlings. It might be because the root was more responsive to the influence of the fungus during co-culture compared to other parts. This needs to be investigated further in future studies.

There have been discrepancies in reports regarding bovine trypsin inhibitory changes during germination of *Phaseolus vulgaris*. Some papers found a decrease (Sathe *et al*., 1983; Sotelo and Lucas, 1998; Sangronis and Machado, 2007; Shimelis and Rakshit, 2007) in the activity and others reported an increase or staying at a constant level during seed germination (Nielsen and Liener, 1988; Savelkoul *et al*., 1992). In the present study, bovine trypsin inhibitory specific activity in red kidney bean cotyledons increased up to 2 days but decreased subsequently and stayed at a constant level up to 11 days. Different conditions of germination including moisture and temperature might lead to these discordant results. Moreover, different extraction methods used by different researchers can remove different isoinhibitors or even proteins during extraction. This could have an impact

on specific trypsin inhibitory activity detected. In the method used in this project, heat-labile proteins were removed before the assay of trypsin inhibitory activity. A constant level of specific trypsin inhibitory activity in the cotyledons of *Cicer arietinum* during 6 days of germination has been reported (Muzquiz *et al*., 2004). In raw dry Indian bean (*Dolichos lablab* L.) trypsin inhibitory activity decreased by 51% during 12 h of imbibition (Ramakrishna *et al*., 2006). Interestingly, bovine trypsin inhibitory activity was detected in the seed coat, seed and root exudates, root, hypocotyl and leaves of red kidney bean. There is no prior report regarding this activity in the aforementioned seed/seedling parts of red kidney bean. Trypsin inhibitory activity has been reported in soybean seed coat (Santos *et al*., 2008) and *Amaranthus cruentus* leaves (Ievleva *et al*., 2000). Expression of the kunitz trypsin inhibitor gene family in the leaves, roots and stems of soybean has been demonstrated (Rashed *et al*., 2008).

UV irradiation was found to increase trypsin inhibitory activity in seed and seedling parts of red kidney bean after 2 days of germination. Induction of trypsin inhibitory activity in common bean by water stress has been reported before (Piergiovanni and Pignone, 2003; Domash *et al*., 2006). Trypsin inhibitory activity has been induced in some plants such as amaranth in response to stress and to abscisic acid (Sanchez-Hernandez *et al*., 2004; Domash *et al*., 2008). There is however, no publication regarding changes in bovine trypsin inhibitor in response of UV irradiation. UV irradiation has also caused protease inhibitor synthesis in tomato leaves (Srinivasan *et al*., 2009). Involvement of protease inhibitors in plant protection under stress has been elucidated before (Dunaevskii *et al*., 2005b). The mechanism by which

co-culturing red kidney bean seedlings with *A. alternata* increased trypsin inhibitory activity in the seed coat, seed and root exudates, root, hypocotyl and leaves but not the cotyledons or embryonic axis is not presently known. Further investigation is needed on this problem. Future work can also focus on purification of trypsin inhibitors induced in response to stress and to compare their structures with those present before induction.

All parts of red kidney bean seedlings as well as root and seed exudates had porcine amylase stimulatory activity. In previous reports PHA has been considered to play a role in plant defence. It has been detected in the embryonic axis, leaves, root, hypocotyl and root exudates (Borrebaeck and Mattiasson, 1983; Hoffman and Donaldson, 1985; Kjemtrup *et al*., 1995; Kovalchuk, 2006). Commercial PHA-L, the AFP purified in this study and mannose binding lectins were all found to have amylase stimulatory activity. In crude extracts of red kidney bean seeds these and possibly other unidentified proteins could account for the observed amylase stimulatory activity. Further work can focus on isolation and identification of other amylase stimulators for comparison of their modes of action. No amylase inhibitor was found in red kidney bean seed coat, root, seed and root exudates, hypocotyl and leaves using a gel electrophoresis method. This is in agreement with a previous report which assessed amylase inhibitory activity in common beans using an immunoblotting method and partial purification of the inhibitors (Moreno *et al*., 1990). In the cotyledons, amylase inhibitory activity was present up to two days of germination and afterwards the stimulator masked it even though based on electrophoresis analysis amylase

isoinhibitors were clearly present in the cotyledonary extracts until day 11 of germination.

As there was amylase stimulatory activity in red kidney bean cotyledons and embryonic axis, it was difficult to assess changes in amylase inhibitory activity in response to UV irradiation, but our preliminary observation based on in-gel electrophoresis showed that polypeptides with less and weaker isoinhibitor activity were present in the cotyledons or embryonic axis treated with UV irradiation. This is consistent with a previous work showing less amylase inhibitory activity in chickpeas meal treated with UV irradiation (Mulimani and Rudrappa, 1994). Future work can be carried out with other techniques such as Western blotting or gene expression to investigate changes in amylase inhibitory activity after exposure to UV irradiation. Amylase stimulators and specifically PHA can be found in root and seed exudates (Kjemtrup *et al*., 1995; Kovalchuk, 2006). As PHA is structurally similar to amylase inhibitors (Mirkov *et al*., 1994), it is interesting that the latter was not present in root exudates. Leaching of PHA from bean seeds to the outside has been considered as a defence mechanism of germinated seeds because of the action of lectin against microorganisms (Kovalchuk, 2006). Interestingly, UV irradiation caused less stimulatory activity in extracts of the seed and root exudates, root and hypocotyl. As several proteins might be involved in amylase stimulatory activity, it is not clear why there was a reduction of amylase stimulatory activity in these extracts.

Abscisic acid has been reported as an inducer of α-amylase inhibitor (Domash *et al*., 2006). It has also induced an inhibitor against insect α-

amylase in *Amaranthus hypochondriacus* leaves (Sanchez-Hernandez *et al*., 2004). As *A. alternata* has been reported as a producer of ABA, induction of α-amylase inhibitor by ABA in red kidney bean seedlings co-cultured with the fungus seems likely. Further investigation is needed in future using the Western blotting technique, for example.

Co-culturing of red kidney bean seeds with *A. alternata* caused lower amylase stimulatory activity in the embryonic axis, seed and root exudates, root and hypocotyl. The influence of the fungus on this and other changes in red kidney bean seedlings during co-culture might be mediated through volatiles, diffusible metabolites or pH changes in the medium, etc.

Chitinase was detected in red kidney bean seed/seedling parts including the cotyledons, embryonic axis, seed coat, root, hypocotyls, leaves as well as root and seed exudates. This has been reported in seed parts of *Phaseolus vulgaris* (Ramos *et al*., 1998; Zhou *et al*., 2002) and bean leaves, hypocotyl and roots treated with ethylene or either pathogenic or non-pathogenic fungi (Vogeli *et al*., 1988; Benhamou *et al*., 1993; Dann *et al*., 1996; Lang *et al*., 1996; Xue *et al*., 1998; Lima *et al*., 2005; Campos *et al*., 2009). These results are also consistent with other reports showing presence of chitinases in seed and seedling parts of uninfected plants including pea, winged bean, tobacco, mung bean, *Arabidopsis* sp. and *Adenanthera pavonina* (Petruzzelli *et al*., 1999; Lima *et al*., 2002; Sekeli *et al*., 2003; Ye and Ng, 2005; Santos *et al*., 2007). Chitinase has been detected in root and seed exudates of two legumes including cowpea and *Adenanthera pavonina* (Santos *et al*., 2004; Noberga *et al*., 2005) but its occurrence in seed and root exudates of red

kidney bean has not bean reported before. The enzyme activity was increased in all seed and seedling parts by the UV irradiation treatment. Induction of chitinase by UV in bean leaves is consistent with a previous report (Margis-Pinheiro *et al*., 1993). Induction of chitinase in the fruit of tomatoes and peaches by UV-C has also been demonstrated in a previous report (Ghaouth *et al*., 2003; Charles *et al*., 2009).

Co-culturing of red kidney bean seeds with *A. alternata* induced chitinase activity in the seed coat, seed and root exudates, root and leaves. This is consistent with previous reports showing induction of chitinase in seedling parts by biotic stress factors (Dann *et al*., 1996; Lang *et al*., 1996; Xue *et al*., 1998; Campos *et al*., 2009).

For the first time β-1, 3- glucanase activity was found in seed and root exudates of red kidney bean. Presence of this enzyme activity has also been shown in the seed and root exudates of two other legumes (*Adenanthera pavonina* L.) and cowpea (Santos *et al*., 2004; Nobrega *et al*., 2005). The enzyme activity has been found in pea embryonic axis as well as cotyledons which increased during germination (Petruzzelli *et al*., 1999). The finding that the seed coat, root and hypocotyl of red kidney bean had this enzyme activity is consistent with that of another study on the pea (Buchner *et al*., 2002).

The enzyme activity was also detected in red kidney bean leaves but UV treatment had no effect on the enzyme activity. This is different from a previous report showing induction of class I glucanase gene in bean leaves

(Kucera *et al*., 2003) but similar to other reports (Brederode *et al*., 1991; Jung *et al*., 1995; Thalmair *et al*., 1996). There have been some discrepancies in findings about class I glucanase induction by UV irradiation.

UV irradiation induced the activity in the cotyledons and root but not other parts of red kidney bean seedlings. Induction of β-1, 3- glucanase in the fruit of tomatoes and peaches by UV-C has been demonstrated in previous reports (Ghaouth *et al*., 2003; Charles *et al*., 2009).

Co-culturing of the seeds with the fungus increased β-1, 3- glucanase activity only in red kidney bean leaves. Induction of β-1, 3- glucanase in bean leaves and hypocotyl by biotic agents or ethylene has been reported before (Vogeli *et al*., 1988; Hughes and Dickerson, 1991; Daugrois *et al*., 1992; Dann *et al*., 1996; Campos *et al*., 2009).

In conclusion, AF activity against *A. alternata* in response to the UV irradiation treatment and co-culturing of red kidney bean seedlings with the fungus was not associated with changes in inhibitory activities of amylase and trypsin as well as that of chitinase and β-1, 3- glucanase. This is consistent with previous results regarding absence of these bioactivities in the purified AFP (see chapter 3).

Chapter 8- Summary, Conclusions and Future Directions

8.1- Summary and conclusions

Leguminous plants contain bioactive proteins including lectins, hydrolytic enzymes, proteinaceous enzyme inhibitors and antifungal proteins which can affect organisms. In this project low-pH and heat-stable bioactive proteins including trypsin inhibitors, α-amylase inhibitors, chitinases and β-1, 3-glucanases present in red kidney bean seeds were studied and growth inhibitory activity of seed extracts containing these bioactive proteins against a model fungus *Alternaria alternata* was determined.

A protein with antifungal activity against *A. alternata* was purified from red kidney bean cotyledons and embryonic axis using different column chromatographic procedures. An AFP was purified and found to be a fungistat as its toxicity was reversible. Morphological changes and poor development of mycelia treated with the AFP were observed. Its N-terminal sequence was identical to an antifungal protein previously isolated from flageolet beans (Xia and Ng, 2005). The protein was found to be devoid of inhibitory activities against following enzymes: bovine trypsin, porcine pancreatic α-amylase (PPA), protease and amylase of *A. alternata*. Instead, it was able to enhance PPA activity. Also it did not possess chitinase or β-1, 3-glucanase activity. Taken together these results suggest that the mode of action by which this AFP inhibits growth of *A. alternata* is not related to the four bioactive proteins.

Crude red kidney bean seed extracts inhibited bovine trypsin and protease of *A. alternata*. Although porcine α-amylase activity was also inhibited, the activity of amylases of *A. alternata* and *Aspergillus oryzae* were not, suggesting that there might be different interactions between the bean enzyme inhibitor and different amylases. The purified AFP inhibited growth of *A. alternata* but did not inhibit its protease activity suggesting the fungal protease inhibitor present in crude red kidney bean seed extracts and the purified AFP could be two distinct proteins.

A preliminary observation showed induced melanization and conidiation in *A. alternata* in response to added crude seed extracts. With the aid of MALDI tandem Time-of-Flight analysis (MALDI TOF/TOF), a previously identified mannose-binding lectin (MBL) or PvFRIL (*Phaseoulus vulgaris* fetal liver tyrosine kinase 3-receptor interacting lectin) has been found to have an unrecognised bioactivity until now. To better assess the conidiogenic effect of the MBL a method was developed based on small PDA discs as other established methods were not appropriate to be used for exogenous application of a protein. The conidia produced in response to the MBL were similar to those induced by other methods and their germ tubes were longer after 12 h growth than those induced under white light (control).

Two diametrically opposite activities, namely porcine α-amylase inhibitors and stimulators, were detected in red kidney bean seeds. Both activities were present in the cotyledons and embryonic axis. However, in the cotyledons amylase inhibitory activity was stronger and masked amylase stimulatory activity. In the embryonic axis, the reverse was observed. When cotyledonary extracts were boiled for 10 min, amylase inhibitors were

inactivated to allow detection of amylase stimulatory activity. Phytohemagglutinins including PHA-L were responsible for amylase stimulatory activity although other proteins including the AFP and MBL purified in this study were also found to possess this activity. Almost all seedling parts were found to possess amylase stimulatory activity. Using an in-gel electrophoretic method presence of porcine α-amylase (PPA) inhibitory activity in the cotyledons and embryonic axis but not all other seed and seedling parts was shown. Interestingly, no stimulatory activity was detected when bean seed extracts were mixed with amylases of *Aspergillus oryzae* and *A. alternata* suggesting that interaction between the stimulator in seed extracts and PPA was specific.

In this research chitinase and β-1, 3- glucanase activities were detected in red kidney bean seed parts but no connection was found between AF activity against *A. alternata* and these activities. The levels of these enzyme activities varied in different seed parts.

An improved method to detect trypsin inhibitors in plant extracts after gel electrophoresis was developed. Azoalbumin, a coloured protein, was immobilized (with a concentration no lower than 3%) in non-denaturing polyacrylamide gels for separation of protease inhibitors in seed extracts. Bands on a gel that were retained after digestion with added trypsin showed presence of trypsin inhibitors. This simplifies the procedure to detect trypsin isoinhibitors after gel electrophoresis.

Some proteins were found to possess more than one function in this project. PHA including PHA-L and PvFRIL showed bovine trypsin inhibitory and porcine α-amylase stimulatory activities. PvFRIL was also able to induce conidiation in *A. alternata*.

Changes in several bioactivities in red kidney bean seed, seedling parts and pods during seed development were studied. Additionally, changes in the selected bioactivities during seed germination with or without influence of biotic (co-culture with *A. alternata*) or abiotic (UV irradiation) stressors were also studied. No AF activity against *A. alternata* was found in the pods and seed coat during seed formation. AF activity was observed in the seed coat during germination suggesting presence of AFPs therein after the start of imbibition. Bovine trypsin inhibitory activity was observed in all the seed parts and pods during seed development. The highest trypsin inhibitory activity was found in the cotyledons and embryonic activity at day 30 and 35 after flower fall, respectively. Most bioactive proteins studied were found to accumulate in the seeds at 20 d after flower fall and their levels decreased during 11 days of germination. The bioactivities of these proteins decreased in pods as they were losing water and showed visible signs of ageing.

The changes in some bioactivities in several seed and seedling parts, seed and root exudates are reported here for first time. Moreover, their changes under stress and during seed development have not been investigated before.

The overall changes in AF activity against *A. alternata* during seed development and germination of red kidney bean did not mirror those of the bioactive proteins including chitinase and β-1, 3- glucanase. This is consistent with the result that the purified AFP did not show these activities. UV irradiation as well as co-culture of the seeds with *A. alternata* increased

some of these bioactivities in some seed or seedling parts. These may be related to stress responses.

8.2- Future studies

A number of further studies can be pursued based on the results obtained. Some of these are listed below.

1. Other techniques including Western blotting using antibodies against the purified bioactive proteins of interest are recommended for further investigations into their changes during seed development and germination. They are particularly useful in case that different proteins showed the same bioactivities. Both PHA and MBA, for example, showed trypsin inhibitory and porcine α-amylase stimulatory activities in *in vitro* experiments.

2. Purification and identification of AFPs that might be responsible for the AF activity detected in the different seed/seedlings parts.

3. It is important to study mechanisms of actions of AFPs before using them in the food industry or control of pathogenic fungi. There is a dearth of information in the literature on mechanisms of action of AFPs and this is why they have not been used widely (Theis *et al*., 2005). More microscopic studies including transmission electron microscopy (for example, using immunogold-labeled AFP) and confocal laser scanning microscopy (for example, using labeled-protein) can be carried out to find out more about internalization and localization of AFP into fungal cells and ultrastructural changes in the

fungus such as increased vacuolization or plasma membrane permeabilization.

4. It would be of interest to determine if the extracts from bean seed parts containing stimulatory activity towards porcine amylase could also have a similar effect on the human salivary enzyme. Some commercial starch-blockers derived from *Phaseolus vulgaris* seeds are used as an obesity reducer (Mosca *et al*., 2008). These blockers have been claimed to inhibit digestion of complex carbohydrates such as starch.

5. It is not known how a protein can stimulate the activity of an amylase. Some insights about this might be gained by an investigation into the molecular interactions of these proteins.

6. Isolation and identification of other proteins besides the lectins studied here would enable comparative studies and gain insights into their mechanisms of action.

7. MBL can be assessed for its conidiogenic effect on other fungi. Other fungi including those which produce pycnidia or acervuli would be very interesting for this study. Other proteins from plants can be assessed for their potential on fungal conidiation. Melanin biosynthesis inhibitors such as glyphosate, tricyclazole, PCBA, pyroqilon, coumarin, phthalide, tetrachlorophthalide can be co-applied with MBL to see their effect on melanin and conidia production.

8. Some bioactivities including trypsin inhibitor, chitinase and β-1, 3-glucanase activities were increased under UV stress or co-culturing with *A. alternata*. These induced proteins can be purified and compared with the constitutive bioactive proteins present in the seed to see whether they are structurally the same or not.

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Appendices

N-TERMINAL SEQUENCING REPORT

11 June, 2010 CONFIDENTIAL

Analysis performed by: Georgina Giannikopoulos

Checked and reported by: Bernie McInerney

Customer:
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Sample Details & ID:

One gel sample labelled, **Bean Protein**, was supplied for analysis.

Analysis Procedure:

The protein in the gel band was passively eluted from the gel matrix using SDS elution buffer overnight. The sample was then loaded onto Prosorb filter cartridges (Applied Biosystems) and washed with 0.1 % TFA (2 × 100 µL) to remove the SDS and reduce the background contamination. The sample now on the PVDF membrane was subjected to 8 cycles of Edman N-terminal sequencing.

Automated Edman degradation was carried out using an Applied Biosystems 494 Procise Protein Sequencing System. Performance of the sequencer is assessed routinely with 10 pmol β-Lactoglobulin standard. A copy of sample chromatograms can be supplied if requested.

Our sequence no.: 3117

Project no.: 10188

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Summary of Analysis

The summary of analysis contains information about the completed work and the invoice. An invoice will be forwarded independently to the address given below. Where appropriate please forward this summary to your supervisor or the responsible person if the supervisor's E-mail address differs from the E-mail address given in "User Details".

Job Info

Job ID	08106	Date in	3/09/2008
Project ID	p0	Date out	16/09/2008

User Details

Client	David Leung
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Supervisor	
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Notification of publications containing data obtained from CPR

To report the extent of usage and benefits of the Centre for Protein Research we would like to ask the service users to notify us of any publications containing results obtained from our facility.

The CPR strongly supports projects of undergraduate and postgraduate students. Accordingly we also like to keep records of analyses performed at the CPR that significantly contributed to these projects and were included in reports or theses.

Job ID 08083

Procedure

The procedure described below is a general procedure for the identification of proteins by MALDI tandem Time-of-Flight mass spectrometry. The applied procedure can differ slightly from the example below. Please contact us if you need further details.

Sample preparation
Excised protein spots/bands were subjected to in-gel digestion with trypsin essentially according to the method of Shevchenko et al. (Proc Natl Acad Sci U S A 93, 14440-14445, 1996). Eluted peptides were dried using a centrifugal concentrator. The protocol for tryptic digestion can be downloaded at http://www.biochem.otago.ac.nz/cpr/protocols.html. Peptides were re suspended in 30% [v/v] ACN (acetonitrile) and 0.1% [v/v] TFA (trifluoroacetic acid) in water. One ul of peptide solution was premixed with 2 ul of matrix (10 mg per ml alpha cyano-4-hydroxycinnamic acid (CHCA) dissolved in 65% [v/v] aqueous acetonitrile containing 0.1% [v/v] TFA and 10 mM ammonium dihydrogen phosphate). 0.8 ul of sample/matrix mixture were spotted onto a MALDI sample plate (Opti-TOF 384 well plate, Applied Biosystems, MA) and air dried.
Mass Spectrometry
Samples were analysed on a 4800 MALDI tandem Time-of-Flight Analyzer (MALDI TOF/TOF, Applied Biosystems, MA). All MS spectra were acquired in positive-ion mode with 800-1000 laser pulses per sample spot. The 15 - 20 strongest precursor ions of each sample spot were used for MS/MS collision-induced dissociation (CID) analysis. CID spectra were acquired with 2000-4000 laser pulses per selected precursor using the 2 kV mode and air as the collision gas at a pressure of 2*E-7 torr.
Data Analysis
For protein identification MS/MS data was searched against the UniProt/SWISS-PROT amino acid sequence database (downloaded in January 2006) using the Mascot search engine (http://www.matrixscience.com). The search was set up for full tryptic peptides with a maximum of 4 missed cleavage sites. Carboxyamidomethyl cysteine, oxidized methionine, pyroglutamate (E, Q) were included as variable modifications. The precursor mass tolerance threshold was 75 ppm and the max. fragment mass error 0.3 Da.

Results

Results have been discussed with user and/or have been forwarded via email to david.leung@canterbury.ac.nz

Please contact CPR if hard copies are required.
Raw data is available at CPR if required for further data analyses.

Notes

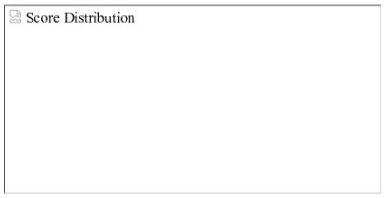
P05087|PHAL_PHAVU Leucoagglutinating phytohemagglutinin precursor - Phaseolus vulgaris (Kidney bean) (French bean)

Mascot Search Results

```
User            : Torsten Kleffmann
Email           : cpr@otago.ac.nz
Search title    : Project: CPR_PMF\Jobs_2008_C, Spot Set: CPR_PMF\Jobs_2008_C\p0_08_M
MS data file    : 08083_aliquot.txt
Database        : UniSprot 1 (359942 sequences; 129199355 residues)
Timestamp       : 5 Aug 2008 at 00:20:59 GMT
Significant hits: P05087|PHAL_PHAVU  Leucoagglutinating phytohemagglutinin precursor - Phaseolus vulgaris (Kidney bean) (French bean)
```

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 40 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As: Peptide Summary — Help

Significance threshold p< 0.05 — Max. number of hits 20

Standard scoring / MudPIT scoring — Ions score cut-off 28 — Show sub-sets

Show pop-ups / Suppress pop-ups — Sort unassigned: Decreasing Score — Require bold red

Select All | Select None | Search Selected | Error tolerant | Archive Report

1. P05087|PHAL_PHAVU **Mass:** 29538 **Score:** 821 **Queries matched:** 12
   Leucoagglutinating phytohemagglutinin precursor - Phaseolus vulgaris (Kidney bean) (French bean)
   Check to include this hit in error tolerant search or archive report

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
11	1106.5654	1105.5581	1105.5363	0.0218	0	74	3.3e-005	1	R.DASVSSSGQLR.L
12	1123.6453	1122.6380	1122.6145	0.0235	0	73	2.8e-005	1	R.HIGIDVNSIR.S
18	1324.7090	1323.7017	1323.6922	0.0096	0	(29)	0.83	1	K.TSFIVSDTVDLK.S
19	1324.7186	1323.7113	1323.6922	0.0192	0	53	0.0036	1	K.TSFIVSDTVDLK.S
25	1753.8638	1752.8565	1752.8318	0.0247	0	91	5e-007	1	K.GNVETNDVLSWSFASK.L
29	1959.0631	1958.0558	1958.0320	0.0239	0	123	3.7e-010	1	K.LSDGTTSEGLNLANLVLNK.I
30	1992.0673	1991.0600	1991.0363	0.0237	0	128	1.2e-010	1	K.SVLPEWVSVGFSATTGINK.G
32	2185.2268	2184.2195	2184.2001	0.0194	1	137	1.3e-011	1	K.LSDGTTSEGLNLANLVLNKIL.-
33	2272.1479	2271.1406	2271.1203	0.0204	1	29	1.1	1	R.DASVSSSGQLRLTNLNGNGEPR.V
40	3726.9292	3725.9219	3725.8576	0.0644	1	34	0.23	1	K.SVLPEWVSVGFSATTGINKGNVETNDVLSWSFASK.L
43	3848.8333	3847.8260	3847.7753	0.0507	1	(74)	2.3e-005	1	K.GGFLGLFDGSNSNFHTVAVEFDTLYNKDWDPTER.H
44	3848.8545	3847.8472	3847.7753	0.0719	1	81	4.3e-006	1	K.GGFLGLFDGSNSNFHTVAVEFDTLYNKDWDPTER.H

Mascot Search Results

Protein View

Match to: **gi|6822274** Score: **713**
mannose lectin FRIL [Phaseolus vulgaris]
Found in search of 08106_B3_conc.txt

Nominal mass (M_r): **31083**; Calculated pI value: **5.38**
NCBI BLAST search of gi|6822274 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Phaseolus vulgaris

Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **22%**

Matched peptides shown in **Bold Red**

```
     1 AQSLSFNFTK FDLDQKDLIF QGDATSTNNV LQLTKLDSGG NPVGASVGRV
    51 LFSAPFHLWE NSMAVSSFET NLTIQISTPH PYYAADGFAF FLAPHDTVIP
   101 PNSWGKFLGL YSNVFRNSPT SENQSFGDVN TDSRVVAVEF DTFPNANIDP
   151 NYRHIGIDVN SIKSKETARW EWQNGKTATA RISYNSASKK STVTTFYPGM
   201 EVVALSHDVD LHAELPEWVR VGLSASTGEE KQKNTIISWS FTSSLKNNEV
   251 KEPKEDMYIA NVVRSYTWIN DVLSYISNK
```

Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
135 - 153	2181.0103	2180.0030	2180.0538	-23	0	R.VVAVEFDTFPNANIDPNYR.H (Ions score 173)
135 - 163	3257.5835	3256.5762	3256.6517	-23	1	R.VVAVEFDTFPNANIDPNYRHIGIDVNSIK.S (Ions score 19)
154 - 163	1095.5964	1094.5891	1094.6084	-18	0	R.HIGIDVNSIK.S (Ions score 85)
221 - 231	1077.5221	1076.5148	1076.5350	-19	0	R.VGLSASTGEEK.Q (Ions score 55)
232 - 246	1739.8911	1738.8838	1738.9254	-24	1	K.QKNTIISWSFTSSLK.N (Ions score 130)
234 - 246	1483.7485	1482.7412	1482.7718	-21	0	K.NTIISWSFTSSLK.N (Ions score 85)
234 - 251	2068.0388	2067.0315	2067.0636	-16	1	K.NTIISWSFTSSLKNNEVK.E (Ions score 81)
234 - 254	2422.2043	2421.1970	2421.2540	-24	2	K.NTIISWSFTSSLKNNEVKEPK.E (Ions score 84)

Error Distribution

```
LOCUS       AAF28739                279 aa            linear   PLN 31-JAN-2000
DEFINITION  mannose lectin FRIL [Phaseolus vulgaris].
ACCESSION   AAF28739
VERSION     AAF28739.1  GI:6822274
DBSOURCE    locus AF121458 accession AF121458.1
KEYWORDS    .
SOURCE      Phaseolus vulgaris
  ORGANISM  Phaseolus vulgaris
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            rosids; eurosids I; Fabales; Fabaceae; Papilionoideae; Phaseoleae;
            Phaseolus.
REFERENCE   1  (residues 1 to 279)
  AUTHORS   Moore,J.G., Colucci,G., Fuchs,C.A., Hicklin,D.J., Chrispeels,M.J.
            and Feldman,M.
  TITLE     A new lectin in red kidney bean called PvFRIL stimulates
            proliferation of NIH3T3 cells expressing the Flt3 receptor
  JOURNAL   Unpublished
REFERENCE   2  (residues 1 to 279)
  AUTHORS   Colucci,G. and Chrispeels,M.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-JAN-1999) Biology, University of California San
            Diego, 9500 Gilman Dr, La Jolla, CA 95093, USA
COMMENT     Method: conceptual translation.
FEATURES             Location/Qualifiers
```

Mascot Search Results

Protein View

Match to: **gi|6822274** Score: **597**
mannose lectin FRIL [Phaseolus vulgaris]
Found in search of 08106_B2_conc.txt

Nominal mass (M_r): **31083**; Calculated pI value: **5.38**
NCBI BLAST search of gi|6822274 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Phaseolus vulgaris

Variable modifications: Carbamidomethyl (C),Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **24%**

Matched peptides shown in **Bold Red**

```
     1 AQSLSFNFTK FDLDQKDLIF QGDATSTNNV LQLTKLDSGG NPVGASVGRV
    51 LFSAPFHLWE NSMAVSSFET NLTIQISTPH PYYAADGFAF FLAPHDTVIP
   101 PNSWGKFLGL YSNVFRNSPT SENQSFGDVN TDSRVVAVEF DTFPNANIDP
   151 NYRHIGIDVN SIKSKETARW EWQNGKTATA RISYNSASKK STVTTFYPGM
   201 EVVALSHDVD LHAELPEWVR VGLSASTGEE KQKNTIISWS FTSSLKNNEV
   251 KEPKEDMYIA NVVRSYTWIN DVLSYISNK
```

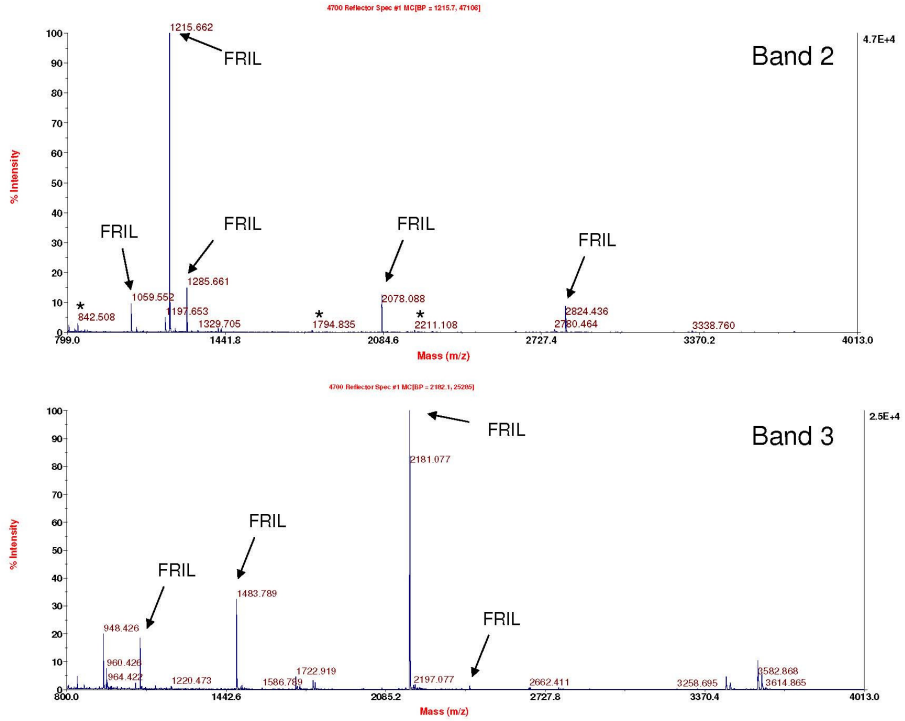
Show predicted peptides also

Sort Peptides By ◉ Residue Number ○ Increasing Mass ○ Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
11 - 35	2824.3933	2823.3860	2823.4291	-15	1	K.FDLDQKDLIFQGDATSTNNVLQLTK.L (Ions score 187)
17 - 35	2078.0486	2077.0413	2077.0692	-13	0	K.DLIFQGDATSTNNVLQLTK.L (Ions score 152)
36 - 49	1285.6371	1284.6298	1284.6422	-10	0	K.LDSGGNPVGASVGR.V (Ions score 129)
107 - 116	1215.6382	1214.6309	1214.6448	-11	0	K.FLGLYSNVFR.N (Ions score 82)
135 - 153	2181.0051	2179.9978	2180.0538	-26	0	R.VVAVEFDTFPNANIDPNYR.H (Ions score 48)

Error Distribution

```
LOCUS       AAF28739                279 aa            linear   PLN 31-JAN-2000
DEFINITION  mannose lectin FRIL [Phaseolus vulgaris].
ACCESSION   AAF28739
VERSION     AAF28739.1  GI:6822274
DBSOURCE    locus AF121458 accession AF121458.1
KEYWORDS    .
SOURCE      Phaseolus vulgaris
  ORGANISM  Phaseolus vulgaris
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; eudicotyledons; core eudicotyledons;
            rosids; eurosids I; Fabales; Fabaceae; Papilionoideae; Phaseoleae;
            Phaseolus.
REFERENCE   1  (residues 1 to 279)
  AUTHORS   Moore,J.G., Colucci,G., Fuchs,C.A., Hicklin,D.J., Chrispeels,M.J.
            and Feldman,M.
  TITLE     A new lectin in red kidney bean called PvFRIL stimulates
            proliferation of NIH3T3 cells expressing the Flt3 receptor
  JOURNAL   Unpublished
REFERENCE   2  (residues 1 to 279)
  AUTHORS   Colucci,G. and Chrispeels,M.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (19-JAN-1999) Biology, University of California San
            Diego, 9500 Gilman Dr, La Jolla, CA 95093, USA
COMMENT     Method: conceptual translation.
FEATURES             Location/Qualifiers
     source          1..279
```



Maldi chromatogram